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GENETIC SEQUENCES AND PROTEINS RELATED TO ALZHEIMER'S DISEASE

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application Serial No. 08/496,841, filed June 28, 1995, which is a continuation-in-part of U.S. Patent Application Serial No. 08/431,048, filed April 28, 1995.

FIELD OF THE INVENTION

The present invention relates generally to the field of neurological and physiological dysfunctions associated with Alzheimer's Disease. More particularly, the invention is concerned with the identification, isolation and cloning of the gene which when mutated is associated with Alzheimer's Disease as well as its transcript, gene products and associated sequence information and neighbouring genes. The present invention also relates to methods of diagnosing for and detection of carriers of the gene, Alzheimer's Disease diagnosis, gene therapy using recombinant technologies and therapy using the information derived from the DNA, protein, and the metabolic function of the protein.

BACKGROUND OF THE INVENTION

In order to facilitate reference to various journal articles, a listing of the articles is provided at the end of this specification.

Alzheimer's Disease (AD) is a degenerative disorder of the human central nervous system characterized by progressive memory impairment and cognitive and intellectual decline during mid to late adult life (Katzman, 1986). The disease is accompanied by a constellation of neuropathologic features principal amongst which are the presence of

extracellular amlyoid or senile plaques and the neurofibrillary degeneration of neurons. The etiology of this disease is complex, although in some families it appears to be inherited as an autosomal dominant trait. However, even among these inherited forms of AD, there are at least three different genes which confer inherited susceptibility to this disease (St. George-Hyslop et al., 1990). The $_e4$ (Cys112Arg) allelic polymorphism of the Apolipoprotein E *(AopE)* gene has been associated with AD in a significant proportion of cases with onset late in life (Saunders et al., 1993; Strittmatter et al., 1993). Similarly, a very small proportion of familial cases with onset before age 65 years have been associated with mutations in the *ß*-amyloid precursor protein (*APP*) gene (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991; Karlinsky et al., 1992; Mullan et al., 1992). A third locus (AD3) associated with a larger proportion of cases with early onset AD has recently been mapped to chromosome 14q24.3 (Schellenberg et al., 1992; St. George-Hyslop et al., 1992; Van Broeckhoven et al., (1992).

Although chromosome 14q carries several genes which could be regarded as candidate genes for the site of mutations associated with AD3 (e.g. *cFOS*, alpha-1-antichymotrypsin, and cathepsin G), most of these candidate genes have been excluded on the basis of their physical location outside the AD3 region and/or the absence of mutations in their respective open reading frames (Schellenberg, GD et al., 1992; Van Broeckhoven, C et al., 1992; Rogaev et al., 1993; Wong et al., 1993).

There have been several developments and commercial directions in respect of treatment of Alzheimer's disease and diagnosis thereof. Published PCT application WO 94 23049 describes transfection of high molecular weight YAC DNA into specific mouse cells. This method is used to analyze large gene complexes, for example the transgenic mice may have increased amyloid precursor protein gene dosage, which mimics the trisomic condition that prevails in Downs Syndrome and the generation of animal models with ß-amyloidosis prevalent in individuals with Alzheimer's Disease. Published international application WO 94 00569 describes transgenic non-human animals harbouring large trans genes such as the trans gene comprising a human amyloid precursor protein gene. Such animal models can provide useful models of human genetic diseases such as Alzheimer's Disease.

Canadian Patent application 2096911 describes a nucleic acid coding for amyloid precursor protein-cleaving protease, which is associated with Alzheimer's Disease and Down's syndrome. The genetic information may be used to diagnose Alzheimer's disease. The genetic information was isolated from chromosome 19. Canadian patent application 2071105, describes detection and treatment of inherited or acquired Alzheimer's disease by the use of YAC nucleotide sequences. The YACs are identified by the numbers 23CB10, 28CA12 and 26FF3.

U.S. Patent 5297562, describes detection of Alzheimer's Disease having two or more copies of chromosome 21. Treatment involves methods for reducing

the proliferation of chromosome 21 trisomy. Canadian Patent Application 2054302, describes monoclonal antibodies which recognize human brain cell nucleus protein encoded by chromosome 21 and are used to detect changes or expression due to Alzheimer's Disease or Down's Syndrome. The monoclonal antibody is specific to a protein encoded by human chromosome 21 and is linked to large pyramidal cells of human brain tissue.

By extensive effort and a unique approach to investigating the AD3 region of chromosome 14q, the Alzheimer's related membrane protein (ARMP) gene has been isolated, cloned and sequenced from within the AD3 region on chromosome 14q24.3. In addition, direct sequencing of RT-PCR products spanning this 3.0 kb cDNA transcript isolated from affected members of at least eight large pedigrees linked to chromosome 14, has led to the discovery of missence mutations in each of these different pedigrees. These mutations are absent in normal chromosomes. It has not been established that the ARMP gene is causative of familial Alzheimer's Disease type AD3. In realizing this link, it is understood that mutations in this gene can be associated with other cognitive, intellectual, or psychological disease such as cerebral hemorrhage, schizophrenia, depression, mental retardation and epilepsy. These phenotypes are present in these AD families and these phenotypes have been seen in mutations of the APP protein gene. The Amyloid Precursor Protein (APP) gene is also associated with inherited Alzheimer's Disease. The identification of both normal and mutant forms of the ARMP gene and gene products has allowed for the

development of screening and diagnostic tests for ARMP utilizing nucleic acid probes and antibodies to the gene product. Through interaction with the defective gene product and the pathway in which this gene product is involved, gene therapy, manipulation and delivery are now made possible.

SUMMARY OF THE INVENTION

Various aspects of the invention are summarized as follows. In accordance with a first aspect of the invention, a purified mammalian polynucleotide is provided which codes for Alzheimer's related membrane protein (ARMP). The polynucleotide has a sequence which is the functional equivalent of the DNA sequence of ATCC deposit 97124, deposited April 28, 1995. The mammalian polynucleotide may be in the form of DNA , genomic DNA, cDNA, mRNA and various fragments and portions of the gene sequence encoding ARMP. The mammalian DNA is conserved in many species, including human and rodents, example, mice. The mouse sequence encoding ARMP has greater than 95% homology with the human sequence encoding the same protein.

Purified human nucleotide sequences which encode mutant ARMP have mutations at nucleotide position i) 685, A→C ii) 737, A→G iii) 986, C→A, iv) 1105, C→G, v) 1478, G→A, vi) 1027, C→T, vii) 1102, C→T and viii) 1422, C→G of Sequence ID No: 1 as well as in the cDNA sequence of a further human clone of a sequence identified by ID NO:133.

The nucleotide sequences encoding ARMP have an alternative splice form in the genes open reading frame. The human cDNA sequence which codes for ARMP has

sequence ID No. 1 as well as sequence SEQ ID NO:133 as sequenced in another human clone. The mouse sequence which encodes ARMP has SEQ ID NO:3, as well as SEQ ID NO:135 derived from a further clone containing the entire coding region. Various DNA and RNA probes and primers may be made from appropriate polynucleotide lengths selected from the sequences. Portions of the sequence also encode antigenic determinants of the ARMP.

Suitable expression vectors comprising the nucleotide sequences are provided along with suitable host cells transfected with such expression vectors.

In accordance with another aspect of the invention, purified mammalian Alzheimer's related membrane protein is provided. The purified protein has an amino acid sequence encoded by polynucleotide sequence as identified above which for the human is SEQ ID NO:2 and SEQ ID NO:134 (derived from another clone). The mouse amino acid sequence is defined by SEQ ID NO:2 and SEQ ID NO.136, the later being translated from another clone containing the entire coding region. The purified protein may have substitution mutations selected from the group consisting of positions identified in SEQ ID NO:2 and Sequence ID NO:134.

i) M 146L

ii) H 163R

iii) A 246E

iv) L 286V

v) C 410 Y

vi) A 260 V

vii) A 285 V

viii) L 392 V

In accordance with another aspect of the invention, are polyclonal antibodies raised to specific predicted sequences of the ARMP protein. Polypeptides of at least six amino acid residues are provided. The polypeptides of six or greater amino acid residues may define antigenic epitopes of the ARMP. Monoclonal antibodies having suitably specific binding affinity for the antigenic regions of the ARMP are prepared by use of corresponding hybridoma cell lines. In addition, other polyclonal antibodies may be prepared by inoculation of animals with suitable peptides or holoprotein which add suitable specific binding affinities for antigenic regions of the ARMP.

In accordance with another aspect of the invention, an isolated DNA molecule is provided which codes for E5-1 protein. A plasmid including this nucleic acid was deposited with the ATCC under the terms of the Budapest Treaty on June 28, 1995 and has been assigned ATCC accession number 97214.

In accordance with another aspect of the invention, purified E5-1 protein is provided, having amino acid SEQ ID NO:138.

In accordance with another aspect of the invention a bioassay is provided for determining if a subject has a normal or mutant ARMP, where the bioassay comprises

providing a biological sample from the subject

conducting a biological assay on the sample to detect a normal or mutant gene sequence coding form ARMP, a normal or mutant ARMP amino acid sequence, or a normal or defective protein function.

In accordance with another aspect of the invention, a process is provided for producing ARMP comprising culturing one of the above described transfected host cells under suitable conditions, to produce the ARMP by expressing the DNA sequence. Alternatively, ARMP may be isolated from mammalian cells in which the ARMP is normally expressed.

In accordance with another aspect of the invention, is a therapeutic composition comprising ARMP and a pharmaceutically acceptable carrier.

In accordance with another aspect of the invention, a recombinant vector for transforming a mammalian tissue cell to express therapeutically effective amounts of ARMP in the cells is provided. The vector is normally delivered to the cells by a suitable vehicle. Suitable vehicles include vaccinia virus, adenovirus, adeno associated virus, retrovirus, liposome transport, neuraltripic viruses, Herpes simplex virus and other vector systems.

In accordance with another aspect of the invention, a method of treating a patient deficient in normal ARMP comprising administering to the patient a therapeutically effective amount of the protein targeted at a variety of patient cells which normally express ARMP. The extent of administration of normal ARMP being sufficient to override any effect the presence of the mutant ARMP may have on the patient. As an alternative to protein, suitable ligands and therapeutic agents such as small molecules and other drug agents may be suitable for drug therapy designed to replace the protein and

defective ARMP, displace mutant ARMP, or to suppress its formation.

In accordance with another aspect of the invention an immuno therapy for treating a patient having Alzheimer's Disease comprises treating the patient with antibodies specific to the mutant ARMP to reduce biological levels or activity of the mutant ARMP in the patient. To facilitate such amino acid therapy, a vaccine composition may be provided for evoking an immune response in a patient of Alzheimer's disease where the composition comprises a mutant ARMP and a pharmaceutically acceptable carrier with or without a suitable excipient. The antibodies developed specific to the mutant ARMP could be used to target appropriately encapsulated drugs/molecules, specific cellular/tissue sites. Therapies utilizing specific ligands which bind to normal or wild type ARMP of either mutant or wild type and which augments normal function of ARMP in membranes and/or cells or inhibits the deleterious effect of the mutant protein are also made possible.

In accordance with another aspect of the invention, a transgenic animal model for Alzheimer's Disease which has the mammalian polynucleotide sequence with at least one mutation which when expressed results in mutant ARMP in animal cells and thereby manifests a phenotype. For example, the human Prion gene when overexpressed in rodent peripheral nervous system and muscle cells causes a quite different response in the animal than the human. The animal may be a rodent and is preferably a mouse, but may also be other animals including rat, pig,

Irosophila melanogaster, C. elegans (nematode), all of which are used for transgenic models. Yeast cells can also be used in which the ARMP Sequence is expressed from an artificial vector.

In accordance with another aspect of the invention, a transgenic mouse model for Alzheimer's Disease has the mouse gene encoding ARMP human or murine homologues mutated to manifest the symptoms. The transgenic mouse may exhibit symptoms of cognitive memory or behavioral disturbances. In addition or alternatively, the symptoms may appear as another cellular tissue disorder such as in mouse liver, kidney, spleen or bone marrow or other organs in which the ARMP gene is normally expressed.

In accordance with another aspect of the invention, the protein can be used as a starting point for rationale drug design to provide ligands, therapeutic drugs or other types of small chemical molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Various aspects of the invention are described hereinafter with respect to the drawings wherein:

Figure 1a. Genomic physical and transcriptional map of the AD3 region of chromosome 14. Genetic map inter-marker genetic distances averaged for male and female meiosis are indicated in centiMorgans.

Figure 1b. Is the constructed physical contig map of overlapping genomic DNA fragments cloned into YACs spanning a FAD locus on chromosome 14q.

Figure 1c. Regions of interest within the constructed physical contig map.

Figure 1d. Transcriptional map illustrating physical locations of the 19 independent longer cDNA clones.

Figure 2. Automated florescent chromatograms representative the change in nucleic acids which direct (by the codon) the amino acid sequence of the gene.

(a) Met 146 Leu

(b) His 163 Arg

(c) Ala 246 Glu

(d) Leu 286 Val

(e) Cys 410 Tyr

Figure 3(a). Restriction fragments of M 146 L mutation using BsphI restriction enzyme in AD patients. Absence of a restriction site indicates a mutant allele.

Figure 3(b). Presence of the His 163 Arg mutation detected by NlaIII restriction digestion. Absence of a restriction indicates a mutant allele.

Figure 3(c). Presence of the Ala 246 Glu mutation in AD patients using DdeI restriction enzyme. Presence of the mutant allele leads to restriction.

Figure 3(d). Presence of Cys 410 Tyr mutation in AD patients as assayed using allelle specific oligonucleotides.

Figure 3(e). Presence of Leu286Val mutation in AD patients using PvuII restriction enzyme in AD patients.

Figure 4. RNA blot demonstrating the expression of ARMP protein mRNA in different regions of the brain including amygdala, caudate, corpus callosum, hippocampus, hypothalamus, substantia nigra, subthalamic nucleus and thalamus.

Figure 5. RNA blot demonstrating the expression of ARMP protein mRNA in a variety of tissues including heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas.

Figure 6a. Hydropathy plot of the putative ARMP protein.

Figure 6b. A model for the structural organization of the putative ARMP protein. Roman numerals depict the transmembrane domains. Putative glycosylation sites are indicated as asterisks and most of the phosphorylation sites are located on the same membrane face as the two acidic hydrophillic loops. The MAP kinase site is present at residue 115 and the PKC site at residue 114. FAD mutation sites are indicated by horizontal arrows.

Figure 7 shows transcription of the *E5-1* gene, investigated by hybridization of the *E5-1* cDNA to Northern blots of mRNA from multiple human brain regions (Panel A), and several peripheral tissues (Panel C). In brain, the *E5-1* transcript is of a lower molecular weight and lesser abundance that the ARMP transcript (Panel B)
hybridized to the same blot using identical conditions.

Figure 8 shows the predicted structure of the *E5-1* protein.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

In order to facilitate review of the various embodiments of the invention and an understanding of various elements and constituents used in making the invention and using same, the following definition of terms used in the invention description is as follows:

Alzheimer Related Membrane Protein gene (ARMP gene) - the chromosome 14 gene which when mutated is associated with familial Alzheimer's Disease and/or other inheritable disease phenotypes (e.g., cerebral hemorrhage, mental retardation, schizophrenia, psychosis, and depression). This definition is understood to include the various sequence polymorphisms that exist, wherein nucleotide substitutions in the gene sequence do not affect the essential function of the gene product, as well as functional equivalents of the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:133, SEQ ID NO:3 and SEQ ID NO:135. This term primarily relates to an isolated coding sequence, but can include some or all of the flanking regulatory elements and/or introns. The term ARMP gene includes the gene in other species analogous to the human gene which when mutated is associated with Alzheimer's Disease.

Alzheimer Related Membrane Protein (ARMP) - the protein encoded by the ARMP gene. The preferred source of protein is the mammalian protein as isolated from humans or animals. Alternatively, functionally equivalent proteins may exist in plants, insects and invertebrates (such as *C.elegans*). The protein may be produced by recombinant organisms, or chemically or enzymatically synthesized. This definition is understood to include functional variants such as the various polymorphic forms of the protein wherein amino acid substitutions or deletions within the amino acid sequence do not affect the essential functioning of the protein, or its structure. It also includes functional fragments of ARMP.

Mutant ARMP gene - The ARMP gene containing one or more mutations which lead to Alzheimer's Disease and/or other inheritable disease phenotypes (e.g., cerebral hemorrhage, mental retardation, schizophrenia, psychosis, and depression). This definition is understood to include the various mutations that exist, wherein nucleotide substitutions in the gene sequence affect the essential function of the gene product, as well as mutations of functional equivalents of the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:133, SEQ ID NO:3 and SEQ ID NO:135 (the corresponding amino acid sequences). This term primarily relates to an isolated coding sequence, but also can include some or all of the flanking regulatory elements and/or introns.

Mutant ARMP - a mammalian protein that is highly analogous to ARMP in terms of primary structure, but wherein one or more amino acid deletions and/or substitutions result in impairment of its essential function, so that mammals, especially humans, whose ARMP producing cells express mutant ARMP rather than the normal ARMP, demonstrate the symptoms of Alzheimer's Disease and/or other relevant inheritable phenotypes (e.g. cerebral hemorrhage, mental retardation, schizophrenia, psychosis, and depression).

mARMP gene - mouse gene analogous to the human ARMP gene. Functional equivalent as used in describing gene sequences and amino acid sequences means that a recited sequence need not be identical to the definitive sequence of the Sequence ID Nos but need only provide a sequence which functions biologically and/or chemically the equivalent of the definitive sequence. Hence

sequences which correspond to a definitive sequence may also be considered as functionally equivalent sequence.

mARMP - mouse Alzheimer related membrane protein, analogous to the human ARMP, encoded by the mARMP gene. This definition is understood to include the various polymorphic forms of the protein wherein amino acid substitutions or deletions of the sequence does not affect the essential functioning of the protein, or its structure.

Mutant mARMP - a mouse protein which is highly analogous to mARMP in terms of primary structure, but wherein one or more amino acid deletions and/or substitutions result in impairment of its essential function, so that mice, whose mARMP producing cells express mutant mARMP rather than the normal mARMP demonstrate the symptoms of Alzheimer's Disease and/or other relevant inheritable phenotypes, or other phenotypes and behaviours as manifested in mice.

ARMP carrier - a mammal in apparent good health whose chromosomes contain a mutant ARMP gene that may be transmitted to the offspring and who will develop Alzheimer's Disease in mid to late adult life.

Missense mutation - A mutation of nucleic acid sequence which alters a codon to that of another amino acid, causing an altered translation product to be made.

Pedigree - In human genetics, a diagram showing the ancestral relationships and transmission of genetic traits over several generations in a family.

E5-1 gene - the chromosome 1 gene which shows homology to the ARMP gene and which when mutated is associated with familial Alzheimer's Disease and/or

other inheritable disease phenotypes. This definition is understood to include the various sequence polymorphisms that exist, wherein nucleotide substitutions in the gene sequence do not affect the essential function of the gene product, as well as functional equivalents of the nucleotide SEQ ID NO:137. This term also includes the gene in other species analogous to the human gene described herein.

E5-1 protein - the protein encoded by the *E5-1* gene. This term includes the protein of SEQ ID NO:138 and also functional variants such as the various polymorphic and splice variant forms of the protein wherein amino acid substitutions or deletions within the amino acid sequence do not affect the essential functioning of the protein. The term also includes functional fragments of the protein.

Mutant E5-1 gene - the *E5-1* gene containing one or more mutations which lead to Alzheimer's Disease. This term is understood to include the various mutations that exist, wherein nucleotide substitutions in the gene sequence affect the essential function of the gene product.

Mutant E5-1 protein - a protein analogous to E5-1 protein but wherein one or more amino acid deletions and/or substitutions result in impairment of its essential function such that mammals, especially humans, whose E5-1 producing cells express mutant E5-1 ~~mutantE5-1~~ protein demonstrate the symptoms of Alzheimer's disease.

Linkage analysis-Analysis of co-segregation of a disease trait or disease gene with polymorphic genetic markers of defined chromosomal location.

hARMP gene - Human ARMP gene.

ORF - Open reading frame.

PCR - Polymerase chain reaction.

contig - continuous cloned regions.

YAC - yeast artificial chromosome.

RT-PCR - reverse transcription polymerase chain reaction.

SSR - Simple sequence repeat polymorphism.

The present invention is concerned with the identification and sequencing of the mammalian ARMP gene in order to gain insight into the cause and etiology of familial Alzheimer's Disease. From this information, screening methods and therapies for the diagnosis and treatment of the disease can be developed. The gene has been identified, cDNA isolated and cloned, its transcripts and gene products identified and sequenced. During such identification of the gene, considerable sequence information has also been developed on intron information in the ARMP gene, flanking untranslated information and signal information and information involving neighbouring genes in the AD3 chromosome region. Direct sequencing of overlapping RT-PCR products spanning the human gene isolated from affected members of large pedigrees linked to chromosome 14 has led to the discovery of missense mutation which co-segregate with the disease.

Although it is generally understood that Alzheimer's Disease is a neurological disorder, most

likely in the brain, expression of ARMP has been found in varieties of human tissue such as heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. Although this gene is expressed widely, the clinically apparent phenotype exists in brain although it is conceivable that biochemical phenotypes may exist in these other tissues. As with other genetic diseases such as Huntington's Disease and APP - Alzheimer's, the clinical disease manifestation may reflect different biochemistries of different cell types and tissues (which stem from genetics and the protein). Such findings suggest that AD may not be solely a neurological disorder but may also be a systemic disorder, hence requiring alternative therapeutic strategies which may be targeted to other tissues or organs or generally in addition or separately from neuronal or brain tissues.

The ARMP mutations identified have been related to Alzheimer's Disease pathology. With the identification of sequencing of the gene and the gene product, probes and antibodies raised to the gene product can be used in a variety of hybridization and immunological assays to screen for and detect the presence of either a normal or mutated gene or gene product.

Patient therapy through removal or blocking of the mutant gene product, as well as supplementation with the normal. gene product by amplification, by genetic and recombinant techniques or by immunotherapy can now be achieved. Correction or modification of the defective gene product by protein treatment immunotherapy (using antibodies to the defective protein) or knock-out of the

mutated gene is now also possible. Familial Alzheimer's Disease could also be controlled by gene therapy in which the gene defect is corrected *in situ* or by the use of recombinant or other vehicles to deliver a DNA sequence capable of expressing the normal gene product, or a deliberately mutated version of the gene product whose effect counter balances the deleterious consequences of the disease mutation to the affected cells of the patient.

The present invention is also concerned with the identification and sequencing of a second gene, the *E5-1* gene on chromosome 1, which is associated with familial Alzheimer's Disease.

Disease mechanism insights and therapies analogous to those described above in relation to the ARMP gene will be available as a result of the identification and isolation of the *E5-1* gene.

Isolating the Human ARMP Gene

Genetic mapping of the AD3 locus.

After the initial regional mapping of the AD3 gene locus to 14q24.3 near the anonymous microsatellite markers *D14S43* and *D14S53* (Schellenberg, GD et al., 1992; St. George-Hyslop, P et al., 1992; Van Broeckhoven, C et al., 1992), twenty one pedigrees were used to segregate AD as a putative autosomal dominant trait (St. George-Hyslop P et al., 1992) and to investigate the segregation of 18 additional genetic markers from the 14q24.3 region which had been organized into a high density genetic linkage map (Figure 1b)

(Weissenbach et al., 1992; Gyapay et al., 1994). Pairwise maximum likelihood analyses previously published confirmed substantial cumulative evidence for linkage between FAD and all of these markers (Table 1). However, much of the genetic data supporting linkage to these markers were derived from six large early onset pedigrees FAD1 (Nee et al., 1983) FAD2 (Frommelt et al., 1991), FAD3 (Goudsmit et al., 1981; Pollen, 1993), FAD4 (Foncin et al., 1985) TOR1.1 (Bergamini, 1991) and 603 (Pericak-Vance et al., 1988) each of which provide at lease one anonymous genetic marker from 14q24.3 (St. George-Hyslop, P. et al., 1992).

In order to more precisely define the location of the AD3 gene relative to the known locations of the genetic markers from 14q24.3, recombinational landmarks were sought by direct inspection of the raw haplotype data only from genotyped affected members of the six pedigrees showing definitive linkage to chromosome 14. This selective strategy in this particular instance necessarily discards data from the reconstructed genotypes of deceased affected members as well as from elderly asymptomatic members of large pedigrees, and takes no account of the smaller pedigrees of uncertain linkage status. However, this strategy is very sound because it also avoids the acquisition of potentially misleading genotype data acquired either through errors in the reconstructed genotypes of deceased affected members arising from non-paternity or sampling errors or from the inclusion of unlinked pedigrees.

Upon inspection of the haplotype data for affected subjects, members of the six large pedigrees whose

genotypes were directly determined revealed obligate recombinants at *D14S48* and *D14S53*, and at *D14S258* and *D14S63*. The single recombinant at *D14S53*, which depicts a telomeric boundary for the FAD region, occurred in the same AD affected subject of the FAD1 pedigree who had previously been found to be recombinant at several other markers located telomeric to *D14S53* including *D14S48* (St. George-Hyslop, P et al., 1992). Conversely, the single recombinant at *D14S258*, which marks a centromeric boundary of the FAD region, occurred in an affected member of the FAD3 pedigree who was also recombinant at several other markers centromeric to *D14S258* including *D14S63*. Both recombinant subjects had unequivocal evidence of Alzheimer's Disease confirmed though standard clinical tests for the illness in other affected members of their families, and the genotypes of both recombinant subjects was informative and co-segregating at multiple loci within the interval centromeric to *D14S53* and telomeric to *D14S258*.

When the haplotype analyses were enlarged to include the reconstructed genotypes of deceased affected members of the six large pedigrees as well as data from the remaining fifteen pedigrees with probabilities for linkage of less than 0.95, several additional recombinants were detected at one or more marker loci within the interval between *D14S53* and *D14S258*. Thus, one additional recombinant was detected in the reconstructed genotype of a deceased affected member of each of three of the larger FAD pedigrees (FAD1, FAD2 and other related families), and eight additional

recombinants were detected in affected members of five smaller FAD pedigrees. However, while some of these recombinants might have correctly placed the AD3 gene within a more defined target region, we were forced to regarded these potentially closer "internal recombinants" as unreliable not only of the reasons discussed earlier, but also because they provided mutually inconsistent locations for the AD3 gene within the *D14S53-D14S258* interval.

Construction of a Physical Contig Spanning the AD3 Region.

As an initial step toward cloning the AD3 gene a contig of overlapping genomic DNA fragments cloned into yeast artificial chromosome vectors, phage artificial chromosome vectors and cosmid vectors was constructed (Figure 1b). FISH mapping studies using cosmids derived from the YAC clones 932c7 and 964f5 suggested that the interval most likely to carry the AD3 gene was at least five megabases in size. Because the large size of this minimal co-segregating region would make positional cloning strategies intactable, additional genetic pointers were sought which focused the search for the AD3 gene to one or more subregions within the interval flanked by *D14S53* and *D14S258*. Haplotype analyses at the markers between *D14S53* and *D14S258* failed to detect statistically significant evidence for linkage disequilibrium and/or allelic association between the FAD trait and alleles at any of these markers, irrespective of whether the analyses were restricted to those pedigrees with early onset forms of

FAD, or were generalized to include all pedigrees. This result was not unexpected given the diverse ethnic origins of our pedigrees. However, when pedigrees of similar ethnic descent were collated, direct inspection of the haplotypes observed on the disease bearing chromosomes segregating in different pedigrees of similar ethnic origin revealed two clusters of marker loci (Table 2). The first of these clusters located centromeric to *D14S77 (D14S786*, *D14S277* and *D14S268*) and spanned the 0.95 Mb physical interval contained in YAC 78842 (depicted as region B in figure 1c). The second cluster was located telomeric to *D14S77 (D14S43*, *D14S273*, and *D14S76*) and spanned the ~ 1Mb physical interval included within the overlapping YAC clones 964c2, 74163, 797d11 and part of 854f5 (depicted as region A in figure 1c). Identical alleles were observed in at least two pedigrees from the same ethnic origin (Table 2). As part of the strategy, it was reasoned that the presence of shared alleles at one of these groups of physically clustered marker loci might reflect the co-inheritance of a small physical region surrounding the ARMP gene on the original founder chromosome in each ethnic population. Significantly, each of the shared extended haplotypes were rare in normal caucasian populations and allele sharing was not observed at other groups of markers spanning similar genetic intervals elsewhere on chromosome 14q24.3.

Transcription mapping and preliminary analysis of candidate genes

To isolate expressed sequences encoded within both critical intervals, a direct selection strategy was

used in involving immobilized, cloned, human genomic DNA as the hybridization target to recover transcribed sequences from primary complementary DNA pools derived from human brain mRNA (Rommens et al., 1993). Approximately 900 putative cDNA fragments of size 100 to 600 base pairs were recovered from regions A and B in figure 1c. These fragments were hybridized to Southern blots containing genomic DNAs from each of the overlapping YAC clones and genomic DNAs from humans and other mammals. This identified a subset of 151 clones which showed evidence for evolutionary conservation and/or for a complex structure which suggested that they were derived from spliced mRNA. The clones within this subset were collated on the basis of physical map location, cross-hybridization and nucleotide sequence, and were used to screen conventional human brain cDNA libraries for longer cDNAs. At least 19 independent cDNA clones over 1kb in length were isolated and then aligned into a partial transcription map of the AD3 region (Figure 1d). Only three of these transcripts corresponded to known characterized genes (c*FOS*, dihydrolipoamide succinyl transferase and latent transforming growth factor binding protein 2).

Recovery of Potential Candidate Genes

Each of the open reading frame portions of the candidate genes were recovered by RT-PCR from mRNA isolated from post-mortem brain tissue of normal control subjects and from either post-mortem brain tissue or cultured fibroblast cell lines of affected members of six pedigrees definitively linked to chromosome 14. The RT-PCR products were then screened for mutations using

chemical cleavage and restriction endonuclease fingerprinting single-strand sequence conformational polymorphism methods (Saleeba and Cotton, 1993; Liu and Sommer, 1995), and by direct nucleotide sequencing. With one exception, all of the genes examined, although of interest, were not unique to affected subjects, and did not co-segregate with the disease. The single exception was the candidate gene represented by clone S182 which contained a series of nucleotide changes not observed in normal subjects, but which altered the predicted amino acid sequence in affected subjects. Although nucleotide sequence differences were also observed in some of the other genes, most were in the 3' untranslated regions and none were unique to Ad-affected subjects.

The remaining sequences, a subset of which are mapped in Figure 1b together with additional putative transcriptional sequences not identified in Figure 1c, are identified in the sequence listings as 14 through 43. The SEQ ID NOS:14 to 43 represent neighbouring genes or fragments of neighbouring genes adjacent the hARMP gene or possibly additional coding fragments arising from alternative splicing of the hARMP. SEQ ID NOS:44-126 and SEQ ID NOS:150-160 represent neighboring genomic fragments containing both exon and intron information. Such sequences are useful for creating primers, for creating diagnostic tests, creating altered regulatory sequences and use of adjacent genomic sequences to create better animal models.

Characterization of the hARMP Gene

Hybridization of the S182 clone to northern blots identified a transcript expressed widely in many areas of brain and peripheral tissues as a major 3.0 kb transcript and a minor transcript of 7.0 kb (Figures 4 and 5). Although the identity of the ~ 7.0 kb transcript is unclear, two observations suggest that the ~ 3.0 kb transcript represents an active product of the gene. Hybridization of the S182 clone to northern blots containing mRNA from a variety of murine tissues, including brain, identifies only a single transcript identical in size to the ~ 3.0 kb human transcript. All of the longer cDNA clones recovered to date (2.6-2.8 kb), which include both 5' and 3' UTRs and which account for the ~ 3.0 kb band on the northern blot, have mapped exclusively to the same physical region of chromosome 14. From these experiments the ~ 7.0 kb transcript could represent either a rare alternatively spliced or polyadenylated isoform of the ~ 3.0 transcript or could represent another gene with homology to S182.

The nucleotide sequence of the major transcript was determined from the consensus of eleven independent longer cDNA clones and from 3 independent clones recovered by standard 5' rapid amplification of cDNA ends and bears no significant homology to other human genes. The cDNA of the sequenced transcript is provided in SEQ ID NO:1 and the predicted amino acid sequence is provided in SEQ ID NO:2. The cDNA sequence of another sequenced human clone is also provided as SEQ ID NO:133 and its predicted amino acid sequence is provided in SEQ ID NO:134.

Analysis of the 5' end of multiple cDNA clones and RT-PCR products as well as corresponding genomic clones indicates that the 5' UTR is contained within at least two exons and that transcription either begins from two different start sites and/or that one of the early 5' untranslated exons is alternatively spliced (Table 6). The longest predicted open reading frame contains *467* amino acids with a small alternatively spliced exon of 4 aminc acids at 25 codons from the putative start codon (Table 3). This putative start codon is the first in phase ATG located 63 bp downstream of a TGA stop codon and lacks a classical Kozak consensus sequences around the first two in-phase ATG sequences (Rogaer et al., in preparation). Like other genes lacking classical 'strong' start codons, the putative 5' UTR of the human transcripts are rich in GC.

Comparison of the nucleic acid and predicted amino acid sequences with available databases using the BLAST alignment paradigms revealed modest amino acid similarity with the C. *elegans* sperm integral membrane protein *SPE-4* ($p=1.5e^{25}$, 24-37% identity over three groups of at least fifty residues) and weaker similarity to portions of several other membrane spanning proteins including mammalian chromogranin A and alpha subunit of mammalian voltage dependent calcium channels (Altschul et al., 1990). This clearly established that they are not the same gene. The amino-acid sequence similarities across putative transmembrane domains may occasionally yield alignment that simply arises from the limited number of hydrophobic amino acids, but there is also extended sequence alignment between S182 protein and

SPE-4 at several hydrophillic domains. Both the putative S182 protein and *SPE-4* are predicted to be of comparable size (467 and 465 residues, respectively) and to contain at least seven transmembrane domains with a large acidic domain preceding the final predicted transmembrane domains with a large acidic domain preceding the final predicted transmembrane domain. The S182 protein does have a longer predicted hydrophillic region at the N terminus.

Further investigation of the hARMP has revealed a host of sequence fragments which form the hARMP gene and include intron sequence information, 5' end untranslated sequence information and 3' end untranslated sequence information (Table 6). Such sequence fragments are identified in Sequence ID Nos. 6 to 13.

Mutations in the S182 Transcript

Direct sequencing of overlapping RT-PCR products spanning the 3.0 kb S182 transcript isolated from affected members of the six large pedigrees linked to chromosome 14 led to the discovery of eight missense mutations in each of the six pedigrees (Table 7, Figure 2). Each of these mutations co-segregated with the disease in the respective pedigrees [Figures 3(a)(b)(c)(d)(e)], and were absent from 142 unrelated neurologically normal subjects drawn from the same ethnic origins as the FAD pedigrees (284 unrelated chromosomes).

The location of the gene within the physical interval segregating with AD3 trait, the presence of eight different missense mutations which co-segregate

with the disease train in six pedigrees definitively linked to chromosome 14, and the absence of these mutations in 284 independent normal chromosomes cumulatively confirms that the hARMP gene is the AD3 locus. Further biologic support for this hypothesis arises from the fact that the residues mutated in FAD kindreds are conserved in evolution (Table 3) and occur in domains of the protein which are also highly conserved, and from the fact that the S182 gene product is expressed at high levels in most regions of the brain including the most severely affected with AD.

The DNA sequence for the hARMP gene as cloned has been incorporated into a plasmid Bluescript. This stable vector has been deposited at ATCC under accession number 97124 on April 28, 1995.

Several mutations in the hARMP gene have been identified which cause a severe type of familial Alzheimer's Disease. One, or a combination of these mutations may be responsible for this form of Alzheimer's Disease as well as several other neurological disorders. The mutations may be any form of nucleotide sequence alteration or substitution. Specific disease causing mutations in the form of nucleotide and/or amino acid substitutions have been located, although we anticipate additional mutations will be found in other families. Each of these nucleotide substitutions occurred within the putative ORF of the S182 transcript, and would be predicted to change the encoded amino acid at the following positions, numbering from the first putative initiation codon. The mutations are listed in respect of their

nucleotide locations in SEQ ID NO:1 and SEQ ID NO:133 (an additional human clone) and amino acid locations in SEQ ID NO:2 and SEQ ID NO:134 (the additional human clone).

i)	685, A→C	Met	146	Leu
ii)	737, A→G	His	163	Arg
iii)	986, C→A	Ala	246	Glu
iv)	1105, C→G	Leu	286	Val
v)	1478, G→A	Cys	410	Tyr
vi)	1027, C→T	Ala	260	Val
vii)	1102, C→T	Ala	285	Val
viii)	1422, C→G	Leu	392	Val

The Met146Leu, Ala246Glu and Cys410Tyr mutations have not been detected in the genomic DNA of affected members of the eight remaining small early onset autosomal dominant FAD pedigrees or six additional families in our collection which express late FAD onset. We predict that such mutations would not commonly occur in late onset FAD which has been excluded by genetic linkage studies from the more aggressive form of AD linked to chromosome 14q24.3 (St. George-Hyslop, P et al., 1992; Schellenberg et al., 1993). The His163Arg mutation has been found in the genomic DNA of affected members of one additional FAD pedigree for which positive but significant statistical evidence for linkage to 14 becomes established. Age of onset of affected members was consistent with affected individuals from families linked to chromosome 14.

Mutations Ala260Val, Ala285Val, and Leu392Val all occur within the acidic hydrophilic loop between putative transmembrane 6 (TM6) and transmembrane (TM7)

(Figure 6). Two of the mutations (A260V; A285V) and the L286V mutation are also located in the alternative spliced domain.

All eight of the mutations can be assayed by a variety of strategies (direct nucleotide sequencing, allele specific oligos, ligation polymerase chain reaction, SSCP, RFLPs etc.) using RT-PCR products representing the mature mRNA/cDNA sequence or genomic DNA. Allele specific oligos were chosen for assaying the mutations. For the A260V and the A285V mutations, genomic DNA carrying the exon was amplified using the same PCR primers and methods as for the L286V mutation. PCR products were then denatured and slot blotted to duplicate nylon membranes using the slot blot protocol described for the C410T mutation.

Of all of the nucleotide substitutions co-segregated with the disease in their respective pedigrees (Figures 3a to 3e), none were seen in asymptomatic family members aged more than two standard deviations beyond the mean age of onset, and non were present on 284 chromosomes from unrelated neurologically normal subjects drawn from comparable ethnic origins.

Identification of an Alternative
Splice Form of the ARMP Gene Product

During sequencing studies of RT-PCR products for the ARMP gene recovered from a variety of tissues, it was discovered that some peripheral tissues (principally white blood cells) demonstrated two alternative splice forms of the ARMP gene. One form is identical to the (putatively 467 amino acid) isoform constitutatively expressed in all brain regions. The alternative splice

form results from the exclusion of the segment of the cDNA between base pairs 1018 and 1116 inclusive, and results in a truncated isoform of the ARMP protein wherein the hydrophobic part of the hydrophilic acidically-charged loop immediately C-terminal to TM6 is removed. This alternatively spliced isoform therefore is characterized by preservation of the sequence N-terminal to and including the tyrosine at position 256, changing of the aspartate at 257 to alanine, and splicing on to the C-terminal part of the protein from and including tyrosine 291. Such splicing differences are often associated with important functional domains of the proteins. This argues that this hydrophilic loop (and consequently the N-terminal hydrophilic loop with similar amino acid charge) is/are active functional domains of the ARMP product and thus sites for therapeutic targeting.

ARMP Protein

With respect to DNA SEQ ID NO.1 and DNA SEQ ID NO:133, analysis of the sequence of overlapping cDNA clones predicted an ORF protein of 467 amino acids when read from the first in phase ATG start codon and a molecular mass of approximately 52.6kDa as later described, due to either polymorphisms in the protein or alternate splicing of the transcript, the molecular weight of the protein can vary due to possible substitutions or deletions of amino acids.

The analysis of predicted amino acid sequence using the Hopp and Woods algorithm suggested that the protein product is a multispanning integral membrane protein such as a receptor, a channel protein, or a structural

membrane protein. The absence of recognizable signal peptide and the paucity of glycoslyation sites are noteworthy, and the hydropathy profile suggests that the protein is less likely to be a soluble protein with a highly compact three-dimensional structure.

The protein may be a cellular protein with a highly compact three dimensional structure in which respect is may be similar to APOE which is also related to Alzheimer's Disease. In light of this putative functional role, it is proposed that this protein be labeled as the Alzheimer Related Membrane Protein (ARMP). The protein also contains a number of potential phosphorylation sites, one of which is the consensus site for MAPkinase which is also involved in the hyperphosphorlyation of *tau* during the normal conversion of normal *tau* to neurofibrillary tangles. This consensus sequence may provide a common putative pathway linking this protein and other known biochemical aspects of Alzheimer's Disease and would represent a likely therapeutic target. Review of the protein structure reveals two sequence YTPF (residues 115-119) SEQ ID NO:161 and STPE (residues 353-356) SEQ ID NO:162 which represent the 5/T- P motif which is the MAP kinase consensus sequence. Several other phosphorylation sites exist with consensus sequences for Protein Kinase C activity. Because protein kinase C activity is associated with differences in the metabolism of APP which are relevant to Alzheimer's Disease, these sites on the ARMP protein and homologues are sites for therapeutic targeting.

The N-terminal is characterized by a highly hydrophilic acid charged domain with several potential phosphorylation domains, followed sequentially by a hydrophobic membrane spanning domain of 19 residues; a charged hydrophilic loop, then five additional hydrophobic membrane spanning domains interspersed with short (5-20 residue) hydrophilic domains; an additional larger acidic hydrophilic charged loop, and then at least one and possibly two other hydrophobic potentially membrane spanning domains culminating in a polar domain at the C-terminus (Table 4 and Figure 6B). The presence of seven membrane spanning domains is characteristic of several classes of G-coupled receptor proteins but is also observed with other proteins including channel proteins.

Comparison of the nucleic acid and predicted amino acid sequences with available databases using the BLAST alignment paradigms revealed amino acid similarity with the *C. elegans* sperm integral membrane protein spe-4 and a similarity to several other membrane spanning proteins including mammalian chromogranin A and the α-subunit of mammalian voltage dependent calcium channels.

The similarity between the putative products of the *spe-4* and ARMP genes implies that they may have similar activities. The *SPE-4* protein of *C. elegans* appears to be involved in the formation and stabilization of the fibrous body-membrane organelle (FMBO) complex during spermatogenesis. The FBMO is a specialized Golgi-derived organelle, consisting of a membrane bound vesicle attached to and partly surrounding a complex of parallel protein fibers and may be involved in the

transport and storage of soluble and membrane-bound polypeptides. Mutations in spe-4 disrupt the FBMO complexes and arrest spermatogenesis. Therefore the physiologic function of *spe-4* may be either to stabilize interactions between integral membrane budding and fusion events, or to stabilize interactions between the membrane and fibrillary proteins during the intracellular transport of the FBMO complex during spermatogenesis. Comparable functions could be envisaged for the ARMP. The ARMP could be involved either in the docking of other membrane-bound proteins such as βAPP, or the axonal transport and fusion budding of membrane-bound vesicles during protein transport such as in the golgi apparatus or endosome-lysosome system. If correct, then mutations might be expected to result in aberrant transport and processing of βAPP and/or abnormal interactions with cytoskeletal proteins such as the microtubule-associated protein *Tau*. Abnormalities in the intracellular and in the extracellular disposition of both βAPP and *Tau* are in fact an integral part of the neuropathologic features of Alzheimer's Disease. Although the location of the ARMP mutations in highly conserved residues within conserved domains of the putative proteins suggests that they are pathogenic, at least three of these mutations are conservative which is commensurate with the onset of disease in adult life. Because none of the mutations observed so far are deletions or nonsense mutations that would be expected to cause a loss of function, we cannot predict whether these mutations will have a dominant gain-of-function effect and promote aberrant processing of βAPP or a

dominant loss-of-function effect causing arrest of normal βAPP processing.

An alternative possibility is that the ARMP gene product may represent a receptor or channel protein. Mutations of such proteins have been causally related to several other dominant neurologic disorders in both vertebrate (e.g., Malignant hyperthermia, hyperkalemic periodic paralysis in humans) and in invertebrate organisms (deg-1(d) mutants in *C. elegans*). Although the pathology of these other disorders does not resemble that of Alzheimer's Disease there is evidence for functional abnormalities in ion channels in Alzheimer's Disease. For example, anomalies have been reported in the tetra-ethylammonium-sensitive 113pS potassium channel and in calcium homeostasis. Perturbations in transmembrane calcium fluxes might be especially relevant in view of the weak homology between S182 and the α-ID subunit of voltage-dependent calcium channels and the observations that increases in intracellular calcium in cultured cells can replicate some of the biochemical features of Alzheimer's Disease such as alteration in the phosphorylation of Tau-microtubule-associated protein and increased production of Aβ peptides.

As mentioned purified normal ARMP protein is characterized by a molecular weight of 52.6kDa. The normal ARMP protein, substantially free of other proteins, is encoded by the aforementioned SEQ ID NO:.1 and SEQ ID NO:133. As will be later discussed, the ARMP protein and fragments thereof may be made by a variety of methods. Purified mutant ARMP protein is

characterized by FAD-associated phenotype (necrotic death, apoptic death, granulovascular degeneration, neurofibrillary degeneration, abnormalities or changes in the metabolism of APP, and Ca^{2+}, K^{+} and glucose, and mitochondrial function and energy metabolism neurotransmitter metabolism, all of which have been found to be abnormal in human brain, and/or peripheral tissue cells in subjects with Alzheimer's Disease) in a variety of cells. The mutant ARMP, free of other proteins, is encoded by the mutant DNA sequence.

Description of the *E5-1* gene,
a Homologue of the ARMP Gene

A gene, *E5-1*, with substantial nucleotide and amino acid homology to the ARMP gene was identified by using the nucleotide sequence of the cDNA for ARMP to search data bases using the BLASTN paradigm of Atschul et al., 1990. Three expressed sequence tagged sites (ESTs) identified by accession numbers T03796, R14600, and R05907 were located which had substantial homology ($p < 1.0\ e^{-100}$, greater than 97% identity over at least 100 contiguous base pairs).

Oligonucleotide primers were produced from these sequences and used to generate PCR products by reverse transcriptase PCR (RT-PCR). These short RT-PCR products were partially sequenced to confirm their identity with the sequences within the data base and were then used as hybridization probes to screen full-length cDNA libraries. Several different cDNA's ranging in size from 1Kb to 2.3Kb were recovered from a cancer cell cDNA library (CaCo-2) and from a human brain cDNA library (E5-1, G1-1, cc54, cc32).

The nucleotide sequence of these clones confirmed that all were derivatives of the same transcript (designated *E5-1*). A plasmid including this nucleic acid was deposited with the ATCC under the terms of the Budapest Treaty on June 28, 1995 and has been assigned ATCC accession number 97214.

The gene encoding the *E5-1* transcript mapped to human chromosome 1 using hybrid mapping panels and to two clusters of CEPH Mega YAC clones which have been placed upon a physical contig map (YAC clones 750g7, 921d12 mapped by FISH to 1q41; and YAC clone 787g12 which also contains an EST of the leukemia associated phosphoproteins *(LAP18)* gene which has been mapped to 1p36.1-p35) (data not shown).

Hybridization of the E5-1 cDNA clones to Northern Blots detected an ~2.3 kilobase mRNA band in many tissues including regions of the brain, as well as a ~2,6K.b mRNA band in muscle, cardiac muscle and pancreas (Figure 7).

In skeletal muscle, cardiac muscle and pancreas, the *E5-1* gene is expressed at relatively higher levels than in brain and as two different transcripts of ~2.3Kb and ~2.6Kb. Both of the *E5-1* transcripts have sizes clearly distinguishable from that of the 2.7Kb ARMP transcript, and did not cross-hybridize with ARMP probes at high stringency. The cDNA sequence of the *E5-1* gene is identified as SEQ ID NO.:137.

The longest ORF within the *E5-1* cDNA consensus nucleotide sequence predicts a polypeptide containing 448 amino acids (numbering from the first in-phase ATG

codon which was surrounded by a GCC-agg-GCt-ATG-c Kozak consensus sequence) (SEQ ID NO.:138).

A comparison of the amino acid sequences of hARMP and E5-1 homologue protein are shown in Table 8. Identical residues are indicated by vertical lines. The locations of mutations in the *E5-1* gene are indicated by downward pointing arrows. The locations of the mutations in the hARMP gene are indicated by upward pointing arrows. Putative TM domains are in open ended boxes. The alternatively spliced exons are denoted by superscripted *(E5-1)* or subscripted (hARMP) "*".

BLASTP alignment analyses also detected significant homology with SPE-4 *of C. elegans* (P=3.5e-26; identity=20-63% over five domains of at least 22 residues), and weak homologies to brain sodium channels (alpha III subunit) and to the alpha subunit of voltage dependent calcium channels from a variety of species (P=0.02; identities 20-28% over two or more domains each of at least 35 residues) (Atschul, 1990). These alignments are similar to those described above for the ARMP gene. However, the most striking homology to the E5-1 protein was found with the amino acid sequence predicted for ARMP. ARMP and *E-51* proteins share 63% overall amino acid sequence identity, and several domains display virtually complete identity (Table 8). Furthermore, all eight residues mutated in ARMP in subjects with *AD3* are conserved in the E5-1 protein (Table 8). As would be expected, hydrophobicity analyses suggest that both proteins also share a similar structural organization.

The similarity was greatest in several domains of the protein corresponding to the intervals between transmembrane domain 1 (TM1) and TM6, and from TM7 to the C-terminus of the ARMP gene. The main difference from ARMP is a difference in the size and amino acid sequence of the acidically-charged hydrophilic loop in the position equivalent to the hydrophilic loop between transmembrane domains TM6 and TM7 in the ARMP protein and in the sequence of the N-terminal hydrophilic domains.

Thus, both proteins are predicted to possess seven hydrophobic putative transmembrane domains, and both proteins bear large acidic hydrophilic domains at the N-terminus and between TM6 and TM7 (figs. 6 and 8). A further similarity arose from analysis of RT-PCR products from brain and muscle RNA, which revealed that nucleotides 1153-1250 of the E5-1 transcript are alternatively spliced. These nucleotides encode amino acids 263-296, which are located within the TM6-TM7 loop domain of the putative E5-1 protein, and which share 94% sequence identity with the alternatively spliced residues 257-290 in ARMP.

The most noticeable differences between the two predicted amino acid sequences occur in the amino acid sequence in the central portion of the TM6-TM7 hydrophilic loop (residues 304-374 of ARMP; 301-355 of E5-1), and in the N-terminal hydrophilic domain (Table 8). By analogy, this domain is also less highly conserved between the murine and human ARMP genes (identity=47/60 residues), and shows no similarity with the equivalent region of SPE-4.

A splice variant of the E5-1 cDNA sequence identified as SEQ ID NO:137 has also been found in all tissues examined. This splice variant lacks the triplet ~~tripet~~ GAA at nucleotide positions 1338-1340.

A further variant has been found in one normal individual whose E5-1 cDNA had C replacing T at nucleotide position 626, which does not change the amino acid sequence.

Mutations of the E5-1 Gene Associated with Alzheimer's Disease

The strong similarity between ARMP and the *E5-1* gene product raised the possibility that the *E5-1* gene might be the site of disease-causing mutations in some of a small number of early onset AD pedigrees in which genetic linkage studies have excluded chromosomes 14, 19 and 21. RT-PCR was used to isolate cDNAs corresponding to the E5-1 transcript from lymphoblasts, fibroblasts or post-mortem brain tissue of affected members of eight pedigrees with early onset familial AD (FAD) in which mutations in the β *APP* and ARMP gene had previously been excluded by direct sequencing studies.

Examination of these RT-PCR products detected a heterozygous A→G substitution at nucleotide 1080 in all four affected members of an extended pedigree of Italian origin (Flo10) with early onset, pathologically confirmed FAD (onset=50-70 yrs.). This mutation would be predicted to cause a Met→Val missense mutation at codon 239 (Table 8).

A second mutation (A→T at nucleotide 787) causing a Asn→Ile substitution at codon 141 was found in affected members of a group of related pedigrees of Volga German

ancestry (represented by cell lines AG09369, AG09907, AG09952, and AG09905, Coriell Institute, Camden, NJ). Significantly, one subject (AG09907) was homozygous for this mutation, an observation compatible with the in-bred nature of these pedigrees. Significantly, this subject did not have a significantly different clinical picture from those subjects heterozygous for the Arg14Ile mutation. Neither of the *E5-1* gene mutations were found in 284 normal Caucasian controls nor were they present in affected members of pedigrees with the *AD3* type of AD.

Both of these mutations would be predicted to cause substitutions of residues which are highly conserved within the ARMP/*E5-1* gene family.

The finding of a gene whose product is predicted to share substantial amino acid and structural similarities with the ARMP gene product suggest that these proteins may be functionally related either as independent proteins with overlapping functions but perhaps with slightly different specific activities, as physically associated subunits of a multimeric polypeptide or as independent proteins performing consecutive functions in the same pathway.

The observation of two different missense mutations in conserved domains of the E5-1 protein in subjects with a familial form of AD argues that these mutations are, like those in the ARMP gene, causal to AD. This conclusion is significant because, while the disease phenotypes associated with mutations in the ARMP gene (onset 30-50 yrs., duration 10 years) are subtly different from that associated with mutations in the *E5-*

1 gene (onset 40-70 years; duration up to 20 years), the general similarities clearly argue that the biochemical pathway subsumed by members of this gene family is central to the genesis of at least early onset AD. The subtle differences in disease phenotype may reflect a lower level of expression of the E5-1 transcript in the CNS, or may reflect a different role for the *E5-1* gene product.

By analogy to the effects of ARMP mutations, E5-1 when mutated may cause aberrant processing of APP (Amyloid Precursor Protein) into Aβ peptide, hyperphosphorylation of Tau microtubule associated protein and abnormalities of intracellular calcium homeostasis. Interference with these anomalous interactions provides a potential therapy for AD.

Functional Domains of the ARMP Protein are Defined by Splicing Sites and Similarities within Other Members of a Gene Family

The ARMP protein is a member of a novel class of transmembrane proteins which share substantial amino acid homology. The homology is sufficient that certain nucleotides probes and antibodies raised against one can identify other members of this gene family. The major difference between members of this family reside in the amino acid and nucleotide sequence homologous to the hydrophillic acid loop domain between putative transmembrane 6 and transmembrane 7 domains of the ARMP gene and gene product. This region is alternatively spliced in some non-neural tissues, and is also the site of several pathogenic disease-causing mutations in the ARMP gene. The

variable splicing of this hydrophilic loop, the presence of a high-density of pathogenic mutations within this loop, and the fact that the amino acid sequences of the loop differs between members of the gene family suggest that this loop is an important functional domain of the protein and may confer some specificity to the physiologic and pathogenic interactions which the ARMP gene product undergoes because the N-terminal hydrophilic domain shares the same acidic charge and same orientation with respect to the membrane, it is very likely that these two domains share functionality either in a coordinated (together) or independent fashion (e.g., different ligands or functional properties). As a result everything said about the hydrophilic loop shall apply also to the N-terminal hydrophilic domain.

Knowledge of the specificity of the loop can be used to identify ligands and functional properties of the ARMP gene product (e.g. sites of interactions with APP, cytosolic proteins such as kinases, *Tau*, and MAP, etc.). Soluble recombinant fusion proteins can be made or the nucleotide sequence coding for amino acids within the loop or parts of the loop can be expressed in suitable vectors (yeast-2-hybrid, baculovirus, and phage -display systems for instance), and used to identify other proteins which interact with ARMP in the pathogenesis of Alzheimer's Disease and other neurological and psychiatric diseases. Therapies can be designated to modulate these interactions and thus to modulate Alzheimer's Disease and the other conditions associated with acquired or inherited

abnormalities of the ARMP gene or its gene products. The potential efficacy of these therapies can be tested by analyzing the affinity and function of these interactions after exposure to the therapeutic agent by standard pharmacokinetic measurements of affinity (Kd and Vmax etc.) using synthetic peptides or recombinant proteins corresponding to functional domains of the ARMP gene (or its homologues). An alternate method for assaying the effect of any interactions involving functional domains such as the hydrophilic loop is to monitor changes in the intracellular trafficking and post-translational modification of the ARMP gene by *in-situ* hybridization, immunohistochemistry, Western blotting and metabolic pulse-chase labeling studies in the presence of and in the absence of the therapeutic agents. A third way is to monitor the effects of "downstream" events including (i) changes in the intracellular metabolism, trafficking and targeting of APP and its products; (ii) changes in second messenger event e.g., cAMP, intracellular Ca^{++} protein kinase activities, etc.

Isolation and Purification of the ARMP Protein

The ARMP protein may be isolated and purified by methods selected on the basis of properties revealed by its sequence. Since the protein possesses properties of a membrane-spanning protein, a membrane fraction of cells in which the protein is highly expressed (e.g., central nervous system cells or cells from other tissues) would be isolated and the proteins removed by extraction and the proteins solubilized using a detergent.

Purification can be achieved using protein purification procedures such as chromatography methods (gel-filtration, ion-exchange and immunoaffinity), by high-performance liquid chromatography (RP-HPLC, ion-exchange HPLC, size-exclusion HPLC, high-performance chromatofocusing and hydrophobic interaction chromatography) or by precipitation (immunoprecipitation). Polyacrylamide gel electrophoresis can also be used to isolate the ARMP protein based on its molecular weight, charge properties and hydrophobicity.

Similar procedures to those just mentioned could be used to purify the protein from cells transfected with vectors containing the ARMP gene (e.g., baculovirus system, yeast expression systems, eukaryotic expression systems).

Purified protein can be used in further biochemical analyses to establish secondary and tertiary structure which may aid in the design of pharmaceuticals to interact with the protein, alter protein charge configuration or charge interaction with other proteins, lipid or saccharide moieties, alter its function in membranes as a transporter channel or receptor and/or in cells as an enzyme or structural protein and treat the disease.

The protein can also be purified by creating a fusion protein by legating the ARMP cDNA sequence to a vector which contains sequence for another peptide (e.g., GST-glutathionine succinyl transferase). The fusion protein is expressed and recovered from prokaryotic (e.g., bacterial or

baculovirus) or eukaryotic cells. The fusion protein can then be purified by affinity chromatography based upon the fusion vector sequence. The ARMP protein can then be further purified from the fusion protein by enzymatic cleavage of the fusion protein.

Isolating Mouse ARMP Gene

In order to characterize the physiological significance of the normal and mutant hARMP gene and gene products in a transgenic mouse model it was necessary to recover a mouse homologue of the hARMP gene. We recovered a murine homologue for the hARMP gene by screening a mouse cDNA library with a labelled human DNA probe and in this manner recovered a 2 kb partial transcript (representing the 3' end of the gene) and several RT-PCR products representing the 5' end. Sequencing of the consensus cDNA transcript of the murine homologue revealed substantial amino acid identity. The sequence cDNA is identified in SEQ ID NO:3 and the predicted amino acid sequence is provided in SEQ ID NO:4. Further sequencing of the mouse cDNA transcript has provided the sequence of the complete coding sequence identified as SEQ ID NO:135 and the predicted amino acid sequence from this sequence is provided in SEQ ID NO:136. More importantly, all of the amino acids that were mutated in the FAD pedigrees were conserved between the murine homologue and the normal human variant (Table 3). This conservation of the ARMP gene as is shown in Table 3, indicates that an orthologous gene exists in the mouse (mARMP), and it is now possible to clone mouse genomic libraries using

human ARMP probes. This will also make it possible to identify and characterize the ARMP gene in other species. This also provides evidence of animals with various disease states or disorders currently known or yet to be elucidated.

Transgenic Mouse Model

The creation of a mouse model for Alzheimer's Disease is important to the understanding of the disease and for the testing of possible therapies. Currently no unambiguous viable animal model for Alzheimer's Disease exists.

There are several ways in which to create an animal model for Alzheimer's Disease. Generation of a specific mutation in the mouse gene such as the identified hARMP gene mutations is one strategy. Secondly, we could insert a wild type human gene and/or humanize the murine gene by homologous recombination. Thirdly, it is also possible to insert a mutant (single or multiple) human gene as genomic or minigene cDNA constructs using wild type or mutant or artificial promoter elements. Fourthly, knock-out of the endogenous murine genes may be accomplished by the insertion of artificially modified fragments of the endogenous gene by homologous recombination. The modifications include insertion of mutant stop codons, the deletion of DNA sequences, or the inclusion of recombination elements (lcx p sites) recognized by enzymes such as Cre recombinase.

To inactivate the mARMP gene chemical or x-ray mutagenesis of mouse gametes, followed by fertilization, can be applied. Heterozygous offspring can then be identified by Southern blotting to demonstrate loss of

one allele by dosage, or failure to inherit one parental allele using RFLP markers.

To create a transgenic mouse a mutant version of hRMP or mARMP can be inserted into a mouse germ line using standard techniques of oocyte microinjection or transfection or microinjection into stem cells. Alternatively, if it is desired to inactivate or replace the endogenous mARMP gene, homologous recombination using embryonic stem cells may be applied.

For oocyte injection, one or more copies of the mutant or wild type ARMP gene can be inserted into the pronucleus of a just-fertilized mouse oocyte. This oocyte is then reimplanted into a pseudo-pregnant foster mother. The liveborn mice can then be screened for integrants using analysis of tail DNA for the presence of human ARMP gene sequences. The transgene can be either a complete genomic sequence injected as a YAC, BAC, PAC or other chromosome DNA fragment, a cDNA with either the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

Retroviral infection of early embryos can also be done to insert the mutant or wild type hARMP. In this method, the mutant or wild type hARMP is inserted into a retroviral vector which is used to directly infect mouse embryos during the early stages of development to generate a chimera, some of which will lead to germline transmission. Similar experiments can be conducted in the cause of mutant proteins, using mutant murine or other animal ARMP gene sequences.

Homologous recombination using stem cells allows for screening of gene transfer cells to identify the rare homologous recombination events. Once identified, these can be used to generate chimeras by injection of mouse blastocysts, and a proportion of the resulting mice will show germline transmission from the recombinant line. This methodology is especially useful if inactivation of the mARMP gene is desired. For example, inactivation of the mARMP gene can be done by designing a DNA fragment which contains sequences from a mARMP exon flanking a selectable marker. Homologous recombination leads to the insertion of the marker sequences in the middle of an exon, inactivating the mARMP gene. DNA analysis of individual clones can then be used to recognize the homologous recombination events.

It is also possible to create mutations in the mouse germline by injecting oligonucleotides containing the mutation of interest and screening the resulting cells by PCR.

This embodiment of the invention has the most significant commercial value as a mouse model for Alzheimer's Disease. Because of the high percentage of sequence conservation between human and mouse it is contemplated that an orthologous gene will exist also in many other species. It is thus contemplated that it will be possible to generate other animal models using similar technology.

Screening and Diagnosis for Alzheimer's Disease

General Diagnostic Uses of the ARMP Gene and Gene Product

The ARMP gene and gene products will be useful for diagnosis of Alzheimer's Disease, presenile and senile dementias, psychiatric diseases such as schizophrenia, depression, etc., and neurologic diseases such as stroke and cerebral hemorrhage - all of which are seen to a greater or lesser extent in symptomatic subjects bearing mutations in the ARMP gene or in the APP gene. Diagnosis of inherited cases of these diseases can be accomplished by analysis of the nucleotide sequence (including genomic and cDNA sequences included in this patent). Diagnosis can also be achieved by monitoring alterations in the electrophoretic mobility and by the reaction with specific antibodies to mutant or wild-type ARMP gene products, and by functional assays demonstrating altered function of the ARMP gene product. In addition, the ARMP gene and ARMP gene products can be used to search for inherited anomalies in the gene and/or its products (as well as those of the homologous gene) and can also be used for diagnosis in the same way as they can be used for diagnosis of non-genetic cases.

Diagnosis of non-inherited cases can be made by observation of alterations in the ARMP transcription, translation, and post-translational modification and processing as well as alterations in the intracellular and extracellular trafficking of ARMP gene products in the brain and peripheral cells. Such changes will include alterations in the amount of ARMP messenger RNA and/or protein, alteration in phosphorylation state, abnormal intracellular location/distribution, abnormal extracellular distribution, etc. Such assays will

include: Northern Blots (with ARMP-specific and ARMP non-specific nucleotide probes which also cross-react with other members of the gene family), and Western blots and enzyme-linked immunosorbent assays (ELISA) (with antibodies raised specifically to: ARMP; to various functional domains of ARMP; to other members of the homologous gene family; and to various post-translational modification states including glycosylated and phosphorylated isoforms). These assays can be performed on peripheral tissues (e.g., blood cells, plasma, cultured or other fibroblast tissues, etc.) as well as on biopsies of CNS tissues obtained antimortem or postmortem, and upon cerebrospinal fluid. Such assays might also include in-situ hybridization and immunohistochemistry (to localized messenger RNA and protein to specific subcellular compartments and/or within neuropathological structures associated with these diseases such as neurofibrillary tangles and amyloid plaques).

Screening for Alzheimer's Disease

Screening for Alzheimer's Disease as linked to chromosome 14 may now be readily carried out because of the knowledge of the mutations in the gene.

People with a high risk for Alzheimer's Disease (present in family pedigree) or, individuals not previously known to be at risk, or people in general may be screened routinely using probes to detect the present of a mutant ARMP gene by a variety of techniques. Genomic DNA used for the diagnosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The DNA

may be isolated and used directly for detection of a specific sequence or may be PCR amplified prior to analysis. RNA or cDNA may also be used. To detect a specific DNA sequence hybridization using specific oligonucleotides, direct DNA sequencing, restriction enzyme digest, RNase protection, chemical cleavage, and ligase-mediated detection are all methods which can be utilized. Oligonucleotides specific to mutant sequences can be chemically synthesized and labelled radioactively with isotopes, or non-radioactively using biotin tags, and hybridized to individual DNA samples immobilized on membranes or other solid-supports by dot-blot or transfer from gels after electrophoresis. The presence or absence of these mutant sequences are then visualized using methods such as autoradiography, fluorometry, or colorimetric reaction. Examples of suitable PCR primers which are useful for example in amplifying portions of the subject sequence containing the aforementioned mutations are set out in Table 5. This table also sets out the change in enzyme site to provide a useful diagnostic tool as defined herein.

Direct DNA sequencing reveals sequence differences between normal and mutant ARMP DNA. Cloned DNA segments may be used as probes to detect specific DNA segments. PCR can be used to enhance the sensitivity of this method. PCR is an enzymatic amplification directed by sequence-specific primers, and involves repeated cycles of heat denaturation of the DNA, annealing of the complementary primers and extension of the annealed primer with a DNA polymerase. This results in an exponential increase of the target DNA.

Other nucleotide sequence amplification techniques may be used, such as ligation-mediated PCR, anchored PCR and enzymatic amplification as would be understood by those skilled in the art.

Sequence alterations may also generate fortuitous restriction enzyme recognition sites which are revealed by the use of appropriate enzyme digestion followed by gel-blot hybridization. DNA fragments carrying the site (normal or mutant) are detected by their increase or reduction in size, or by the increase or decrease of corresponding restriction fragment numbers. Genomic DNA samples may also be amplified by PCR prior to treatment with the appropriate restriction enzyme and the fragments of different sizes are visualized under UV light in the presence of ethidium bromide after gel electrophoresis.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. Small deletions may also be detected as changes in the migration pattern of DNA heteroduplexes in non-denaturing gel electrophoresis. Alternatively, a single base substitution mutation may be detected based on differential PCR product length in PCR. The PCR products of the normal and mutant gene could be differentially detected in acrylamide gels.

Nuclease protection assays (S1 or ligase-mediated) also reveal sequence changes at specific location.

Alternatively, to confirm or detect a polymorphism restriction mapping changes ligated PCR, ASO, REF-SSCP chemical cleavage, endonuclease cleavage at mismatch sites and SSCP may be used. Both REF-SSCP and SSCP are mobility shift assays which are based upon the change in conformation due to mutations.

DNA fragments may also be visualized by methods in which the individual DNA samples are not immobilized on membranes. The probe and target sequences may be in solution or the probe sequence may be immobilized. Autoradiography, radioactive decay, spectrophotometry, and fluorometry may also be used to identify specific individual genotypes. Finally, mutations can be detected by direct nucleotide sequencing.

According to an embodiment of the invention, the portion of the cDNA or genomic DNA segment that is informative for a mutation, can be amplified using PCR. For example, the DNA segment immediately surrounding the C410Y mutation acquired from peripheral blood samples from an individual can be screened using the oligonucleotide primers 885 (tggagactggaacacaac) SEQ ID NO:127 and 893 (gtgtggccagggtagagaact) SEQ ID NO:128. This region would then be amplified by PCR, the products separated by electrophoresis, and transferred to membrane. Labelled oligonucleotide probes are then hybridized to the DNA fragments and autoradiography performed.

ARMP Expression

As an embodiment of the present invention, ARMP protein may be expressed using eukaryotic and prokaryotic expression systems. Eukaryotic expression

systems can be used for many studies of the ARMP gene and gene product including determination of proper expression and post-translational modifications for full biological activity, identifying regulatory elements located in the 5' region of the ARMP gene and their role in tissue regulation of protein expression, production of large amounts of the normal and mutant protein for isolation and purification, to use cells expressing the ARMP protein as a functional assay system for antibodies generated against the protein or to test effectiveness of pharmacological agents, or as a component of a signal transduction system, to study the function of the normal complete protein, specific portions of the protein, or of naturally occurring and artificially produced mutant proteins.

Eukaryotic and prokaryotic expression systems were generated using two different classes of ARMP nucleotide cDNA sequence inserts. In the first class, termed full-length constructs, the entire ARMP cDNA sequence is inserted into the expression plasmid in the correct orientation, and includes both the natural 5' UTR and 3' UTR sequences as well as the entire open reading frame. The open reading frames bear a nucleotide sequence cassette which allows either the wild type open reading frame to be included in the expression system or alternatively , a single or a combination of double mutations can be inserted into the open reading frame. This was accomplished by removing a restriction fragment from the wild type open reading frame using the enzymes NarI and PflmI and replacing it

with a similar fragment generated by reverse transcriptase PCR which bears the nucleotide sequence encoding either the Met146Leu mutation or the Hys163Arg mutation. A second restriction fragment was removed from the wild type normal nucleotide sequence for the open reading frame by cleavage with the enzymes PflmI and NcoI and replaced with restriction fragments bearing either nucleotide sequence encoding the Ala246Glu mutation, or the Ala260Val mutation or the Ala285Val mutation or the Leu286Val mutation, or the Leu392Val mutation, or the Cys410Tyr mutation. Finally, a third variant bearing combinations of either the Met146Leu or His163Arg mutations in tandem with the remaining mutations, was made by linking the NarI-PflmI fragment bearing these mutations and the PflmI-NcoI fragments bearing the remaining mutations.

A second variant of cDNA inserts bearing wild type or mutant cDNA sequences was constructed by removing from the full-length cDNA the 5' UTR and part of the 3' UTR sequences. The 5' UTR sequence was replaced with a synthetic oligonucleotide containing a KpnI restriction site and a Kozak initiation site (oligonucleotide 969: ggtaccgccaccatgacagaggtacctgcac, SEQ ID NO:139). The 3' UTR was replaced with an oligonucleotide corresponding to position 2566 of the cDNA and bears an artificial EcoRI site (oligonucleotide 970: gaattcactggctgtagaaaagac, SEQ ID NO:140). Mutant variants of this construct were then made by inserting the same mutant sequences described above at the NarI-PflmI fragment, and at the PsImI-NcoI sites described above.

For eukaryotic expressions, these various cDNA constructs bearing wild type and mutant sequences described above were cloned into the expression vector pZeoSV (invitrogen). For prokaryotic expression, two constructs have been made using the glutathione S-transferase fusion vector pGEX-kg. The inserts which have been attached to the GST fusion nucleotide sequence are the same nucleotide sequence described above (generated with the oligonucleotide primers 969, SEQ ID NO:139 and 970, SEQ ID NO:140) bearing either the normal open reading frame nucleotide sequence, or bearing a combination of single and double mutations as described above. This construct allows expression of the full-length protein in mutant and wild type variants in prokaryotic cell systems as a GST fusion protein which allows purification of the full length protein followed by removal of the GST fusion product by thrombin digestion. The second prokaryotic cDNA construct was generated to create a fusion protein with the same vector, and allows the production of the amino acid sequence corresponding to the hydrophilic acidic loop domain between TM6 and TM7 of the full-length protein, as either a wild type nucleotide sequence (thus a wild type amino acid sequence for fusion proteins) or as a mutant sequence bearing either the Ala285Val mutation, or the Leu286Val mutation, or the Leu392Val mutation. This was accomplished by recovering wild type or mutant sequence from appropriate sources of RNA using the oligonucleotide primers 989: ggatccggtccacttcgtatgctg, SEQ ID NO:141, and 990:

tttttgaattcttaggctatggttgtgttcca, SEQ ID NO:142. This allows cloning of the appropriate mutant or wild type nucleotide sequence corresponding to the hydrophilic acid loop domain at the BamHI and the EcoRI sites within the pGEX-KG vector.

These prokaryotic expression systems allow the holo-protein or various important functional domains of the protein to be recovered as fusion proteins and then used for binding studies, structural studies, functional studies, and for the generation of appropriate antibodies.

Expression of the ARMP gene in heterologous cell systems can be used to demonstrate structure-function relationships. Ligating the ARMP DNA sequence into a plasmid expression vector to transfect cells is a useful method to test the proteins influence on various cellular biochemical parameters. Plasmid expression vectors containing either the entire, normal or mutant human or mouse ARMP sequence or portions thereof, can be used *in vitro* mutagenesis experiments which will identify portions of the protein crucial for regulatory function.

The DNA sequence can be manipulated in studies to understand the expression of the gene and its product, to achieve production of large quantities of the protein for functional analysis, for antibody production, and for patient therapy. The changes in the sequence may or may not alter the expression pattern in terms of relative quantities, tissue-specificity and functional properties. Partial or full-length DNA sequences which encode for the ARMP protein, modified or unmodified, may

be ligated to bacterial expression vectors. *E. coli* can be used using a variety of expression vector systems, e.g., the T7 RNA polymerase/promoter system using two plasmids or by labeling of plasmid-encoded proteins, or by expression by infection with M13 Phage mGPI-2. *E. coli* vectors can also be used with Phage lamba regulatory sequences, by fusion protein vectors (e.g. lacZ and trpE), by maltose-binding protein fusions, and by glutathione-S-transferase fusion proteins, etc., all of which together with many other prokaryotic expression systems are widely available commercially.

Alternatively, the ARMP protein can be expressed in insect cells using baculoviral vectors, or in mammalian cells using vaccinia virus or specialized eukaryotic expression vectors. For expression in mammalian cells, the cDNA sequence may be ligated to heterlogous promoters, such as the simian varus (SV40) promoter in the pSV2 vector and other similar vectors and introduced into cultured eukaryotic cells, such as COS cells to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin and mycophoenolic acid.

The ARMP DNA sequence can be altered using procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences and site-directed sequence alteration with the use of specific oligonucleotides together with PCR.

The cDNA sequence or portions thereof, or a mini gene consisting of a cDNA with an introl and its own promoter, is introduced into eukaryotic expression vectors by conventional techniques. These vectors permit the transcription of the cDNA in eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. The endogenous ARMP gene promoter can also be used. Different promoters within vectors have different activities which alters the level of expression of the cDNA. In addition, certain promoters can also modulate function such as the glucocorticoid-responsive promoter from the mouse mammary tumor virus.

Some of the vectors listed contain selectable markers or neo bacterial genes that permit isolation of cells by chemical selection. Stable long-term vectors can be maintained in cells as episomal, freely replicating entities using regulatory elements of viruses. Cell lines can also be produced which have integrated the vector into the genomic DNA. In this manner, the gene product is produced on a continuous basis.

Vectors are introduced into recipient cells by various methods including calcium phosphate, strontium phosphate, electroporation, lipofection, DEAE dextran, microinjection, or by protoplast fusion. Alternatively, the cDNA can be introduced by infection using viral vectors.

Using the techniques mentioned, the expression vectors containing the ARMP gene or portions thereof can

be introduced into a variety of mammalian cells from other species or into non-mammalian cells.

The recombinant cloning vector, according to this invention, comprises the selected DNA of the DNA sequences of this invention for expression in a suitable host. The DNA is operatively linked in the vector to an expression control sequence in the recombinant DNA molecule so that normal and mutant ARMP protein can be expressed. The expression control sequence may be selected from the group consisting of sequences that control the expression of genes of prokaryotic or eukaryotic cells and their viruses and combinations thereof. The expression control sequence may be selected from the group consisting of the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of the fd coat protein, early and late promoters of SV40, promoters derived from polyoma, adenovirus, retrovirus, baculovirus, simian virus, 3-phosphoglycerate kinase promoter, yeast acid phosphatase promoters, yeast alpha-mating factors and combinations thereof.

The host cell which may be transfected with the vector of this invention may be selected from the group consisting of *E. coli, pseudomonas, bacillus subtillus, bacillus stearothermophilus,* or other bacili; other bacteria, yeast, fungi, insect, mouse or other animal, plant hosts, or human tissue cells.

For the mutant ARMP DNA sequence similar systems are employed to express and produce the mutant protein.

Antibodies to Detect ARMP

Antibodies to epitopes with the ARMP protein can be raised to provide information on the characteristics of the proteins. Generations of antibodies would enable the visualizations of the proteins in cells and tissues using Western blotting. In this technique, proteins are run on polyacrylamide gel and then transferred onto nitrocellulose membranes. These membranes are then incubated in the presence of the antibody (primary), then following washing are incubated to a secondary antibody which is used for detection of the protein-primary antibody complex. Following repeated washing, the entire complex is visualized using colourimetric or chemiluminescent methods.

Antibodies to the ARMP protein also allow for the use of immunocytochemistry and immunofluorescence techniques in which the proteins can be visualized directly in cells and tissues. This is most helpful in order to establish the subcellular location of the protein and the tissue specificity of the protein.

In order to prepare polyclonal antibodies, fusion proteins containing defined portions or all of the ARMP protein can be synthesized in bacteria by expression of corresponding DNA sequences in a suitable cloning vehicle. The protein can then be purified, coupled to a carrier protein and mixed with Freund's adjuvant (to help stimulate the antigenic response by the rabbits) and injected into rabbits or other laboratory animals. Alternatively, protein can be isolated from cultured cells expressing the protein. Following booster injections at bi-weekly intervals, the rabbits or other laboratory animals

are then bled and the sera isolated. The sera can be used directly or purified prior to use, by various methods including affinity chromatography, Protein A-Sepharose, Antigen Sepharose, Anti-mouse-Ig-Sepharose. The sera can then be used to probe protein extracts run on a polyacrylamide gel to identify the ARMP protein. Alternatively, synthetic peptides can be made to the antigenic portions of the protein and used to inoculate the animals.

To produce monoclonal ARMP antibodies, cells actively expressing the protein are cultured or isolated from tissues and the cell membranes isolated. The membranes, extracts or recombinant protein extracts, containing the ARMP protein, are injected in Freund's adjuvant into mice. After being injected 9 times over a three week period, the mice spleens are removed and resuspended in a phosphate buffered saline (PBS). The spleen cells serve as a source of lymphocytes, some of which are producing antibody of the appropriate specificity. These are then fused with a permanently growing myeloma partner cell, and the products of the fusion are plated into a number of tissue culture wells in the presence of a selective agent such as HAT. The wells are then screened to identify those containing cells making useful antibody by ELISA. These are then freshly plated. After a period of growth, these wells are again screened to identify antibody-producing cells. Several cloning procedures are carried out until over 90% of the wells contain single clones which are positive for antibody production. From this procedure a stable line of clones is established which produce the

antibody. The monoclonal antibody can then be purified by affinity chromatography using Protein A Sepharose, ion-exchange chromatography, as well as variations and combinations of these techniques.

In situ hybridization is another method used to detect the expression of ARMP protein. *In situ* hybridization relies upon the hybridization of a specifically labeled nucleic acid probe to the cellular RNA in individual cells or tissues. Therefore, it allows the identification of mRNA within intact tissues, such as the brain. In this method, oligonucleotides corresponding to unique portions of the ARMP gene are used to detect specific mRNA species in the brain.

In this method a rat is anesthetized and transcardially perfused with cold PBS, followed by perfusion with a formaldehyde solution. The brain or other tissues is then removed, frozen in liquid nitrogen, and cut into thin micron sections. The sections are placed on slides and incubated in proteinase K. Following rinsing in DEP, water and ethanol, the slides are placed in prehybridization buffer. A radioactive probe corresponding to the primer is made by nick translation and incubated with the sectioned brain tissue. After incubation and air drying, the labeled areas are visualized by autoradiography. Dark spots on the tissue sample indicate hybridization of the probe with brain mRNA which demonstrates the expression of the protein.

Antibodies may also be used coupled to compounds for diagnostic and/or therapeutic uses such as radionuclides for imaging and therapy and liposomes for

the targeting of compounds to a specific tissue location.

Isolation and Purification of E5-1 protein

The E5-1 protein may be isolated and purified by the types of methods described above for the ARMP protein.

The protein may also be prepared by expression of the E5-1 cDNA described herein in a suitable host. The protein is ~~a~~ preferably expressed as a fusion protein by ligating its encoding cDNA sequence to a vector containing the coding sequence for another suitable peptide, e.g., GST. The fusion protein is expressed and recovered from prokaryotic cells such as bacterial or baculovirus cells or from eukaryotic cells. Antibodies to ARMP, by virtue of portions of amino acid sequence identity with E5-1, can be used to purify, attract and bind to E5-1 protein and vice versa.

Transgenic Mouse Model of E5-1
Related Alzheimer's Disease

An animal model of Alzheimer's Disease related to mutations of the E5-1 gene may be created by methods analogous to those described above for the ARMP gene.

Antibodies

Due to its structural similarity with the ARMP, the E5-1 protein may be used for the development of probes, peptides, or antibodies to various peptides within it which may recognize both the E5-1 and the ARMP gene and gene products, respectively. As a protein homologue for the ARMP, the E5-1 protein may be used as a replacement for a defective ARMP gene

product. It may also be used to elucidate functions of the ARMP gene in tissue culture and vice versa.

Screening for Alzheimer's
Disease Linked to Chromosome 1

Screening for Alzheimer's Disease linked to mutations of the *E5-1* gene may now be conveniently carried out.

General screening methods are described above in relation to the described mutations in the ARMP gene. These described methods can be readily applied and adapted to detection of the described chromosome 1 mutations, as will be readily understood by those skilled in the art.

In accordance with one embodiment of the invention, the Asn141Ile mutation is screened for by PCR amplification of the surrounding DNA fragment using the primers:

1041: 5'-cattcactgaggacacacc (end-labelled) SEQ ID NO:163 and

1042: 5'-tgtagagcaccaccaaga (unlabelled) SEQ ID NO:164.

Any tissue with nucleated cell may be examined. The amplified products are separated by electrophoresis and an autoradiogram of the gel is prepared and examined for mutant bands.

In accordance with a further embodiment, the Met239Val mutation is screened for by PCR amplification of its surrounding DNA fragment using the primers:

1034: 5'-gcatggtgtgcatccact SEQ ID NO:165 and

1035: 5'-ggaccactctgggaggta SEQ ID NO:166.

The amplified products are separated and an autoradiogram prepared as described above to detect mutant bands.

The same primer sets may be used to detect the mutations by means of other methods such as SSCP, chemical cleavage, DGGE, nucleotide sequencing, ligation chain reaction and allele specific oligonucleotides. As will be understood by those skilled in the art, other suitable primer pairs may be devised and used.

In inherited cases, as the primary event, and in non-inherited cases as a secondary event due to the disease state, abnormal processing of E5-1, ARMP, APP or proteins reacting with E5-1, APP or ARMP, may occur. This can be detected as abnormal phosphorylation, glycoslyation, glycation amidation or proteolytic cleavage products in body tissues or fluids, e.g., CSF or blood.

Therapies

An important aspect of the biochemical studies using the genetic information of this invention is the development of therapies to circumvent or overcome the ARMP gene defect, and thus prevent, treat, control serious symptoms or cure the disease. In view of expression of the ARMP gene in a variety of tissues, one has to recognize that Alzheimer's Disease may not be restricted to the brain. Alzheimer's Disease manifests itself as a neurological disorder which in one of its forms is caused by a mutation in the ARMP gene, but such manifest may be caused by mutations in other organ tissues, such as the liver, releasing factors which affect the brain activity and ultimately cause

Alzheimer's Disease. Hence, in considering various therapies, it is understood that such therapies may be targeted at tissue other than the brain, such as heart, placenta, lung, liver, skeletal muscle, kidney and pancreas, where ARMP is also expressed.

The effect of these mutations in E5-1 and ARMP is a gain of novel function which causes aberrant processing of (APP) Amyloid Precursor Protein into Aβ peptide, abnormal phosphorylation homeostasis, and abnormal apoptosis. Therapy to reverse this will be small molecules (drugs) recombinant proteins, etc. which block the aberrant function by altering the structure of the mutant proteins, etc. which block the aberrant function by altering the structure of the mutant protein, enhancing its metabolic clearance or inhibiting binding of ligands to the mutant protein, enhancing its metabolic clearance or inhibiting binding of ligands to the mutant protein, or inhibiting the channel function of the mutant protein. The same effect might be gained by inserting a second mutant protein by gene therapy similar to the correction of the "Deg 1(d)" and "Mec 4(d)" mutations in *C. elegans* by insertion of mutant transgenes. Alternatively over expression of wild type E5-1 protein or wild type ARMP or both may correct the defect. This could be the administration of drugs or proteins to induce the transcription and translation or inhibit the catabolism of the native E5-1 and ARMP proteins. It could also be accomplished by infusion of recombinant proteins or by gene therapy with vectors causing expression of the normal protein at a high level.

Rationale for Therapeutic, Diagnostic, and Investigational Applications of the ARMP Gene and Gene Products as They Relate to the Amyloid Precursor Protein

The Aβ peptide derivatives of APP are neurotoxic (Selkoe et al, 1994). APP is metabolized by passages through the Golgi network and then to secretory pathways via clathrin-coated vesicles with subsequent passage to the plasma membrane where the mature APP is cleaved by α-secretase to a soluble fraction (Protease Nexin II) plus a non-amyloidogenic C-terminal peptide (Selkoe et al. 1995, Gandy et al., 1993). Alternatively, mature APP can be directed to the endosome-lysosome pathway where it undergoes beta and gamma secretase cleavage to produce the Aβ peptides. The phosphorylation state of the cell determines the relative balance of α-secretase (non-amyloidogenic) or Aβ pathways (amyloidogenic pathway) (Gandy et al., 1993). The phosphorylation state of the cell can be modified pharmacologicially by phorbol esters, muscarinic agonists and other agents, and appears to be mediated by cytosolic factors (especially protein kinase C) acting upon an integral membrane protein in the Golgi network, which we propose to the ARMP, and members of the homologous family (all of which carry several phosphorylation consensus sequences for protein kinease C). Mutations in the ARMP gene will cause alterations in the structure and function of the ARMP gene product leading to defective interactions with regulatory elements (e.g., protein kinase C) or with APP, thereby promoting APP to be directed to the amyloidogenic endosome-lysosome pathway.

Environmental factors (viruses, toxins, and aging, etc.) may also have similar effects on ARMP. To treat Alzheimer's Disease, the phosphorylation state of ARMP can be altered by chemical and biochemical agents (e.g. drugs, peptides and other compounds) which alter the activity of protein kinase C and other protein kinase, or which alter the activity of protein phosphatases, or which modify the availability of ARMP to be postranslationally modified. The interactions between kinases and phosphatases with the ARMP gene products (and the products of its homologues), and the interactions of the ARMP gene products with other proteins involved in the trafficking of the APP within the Golgi network can be modulated to decrease trafficking of Golgi vesicles to the endosome-lysosome pathway thereby promoting Aβ peptide production. Such compounds will include: peptide analogues of APP, ARMP, and homologues of ARMP as well as other interacting proteins, lipids, sugars, and agents which promote differential glycosylation of ARMP and its homologues; agents which alter the biologic half-life of messenger RNA or protein of ARMP and homologues including antibodies and antisense oligonucleotides; and agents which act upon ARMP transcription.

The effect of these agents in cell lines and whole animals can be monitored by monitoring: transcription; translation; post-translational modification of ARMP (e.g., phosphorylation or glycoslyation); and intracellular trafficking of ARMP and its homologues through various intracellular and extracellular

compartments. Methods for these studies include Western and Northern blots; immunoprecipitation after metabolic labeling (pulse-chase) with radio-labeled methionine and ATP, and imminohistochemistry. The effect of these agents can also be monitored using studies which examine the relative binding affinities and relative amounts of ARMP gene products in interactions with protein kinease C and/or APP using either standard binding affinity assays or co-precipitation and Western blots using antibodies to protein kinease C, APP or ARMP and its homologues. The effect of these agents can also be monitored by assessing the production of Aβ peptides by ELISA before and after exposure to the putative therapeutic agent (Huang et al., 1993). The effect can also be monitored by assessing the viability of cell lines after exposure to aluminum salts and to Aβ peptides which are through to be neurotoxic in Alzheimer's Disease. Finally, the effect of these agents can be monitored by assessing the cognitive function of animals bearing: their normal genotype at APP or ARMP homologues; bearing human APP transgenes (with or without mutations); or bearing human ARMP transgenes (with or without mutations); or a combination of all of these.

Rationale for Therapeutic, Diagnostic, and Investigational Applications of the ARMP Gene, the *E5-1* Gene and their Products

The ARMP gene product and the *E5-1* gene product have amino acid sequence homology to human ion channel proteins and receptors. For instance, the E5-1 protein shows substantial homology to the human sodium channel

α-subunit (E=0.18, P=0.16, identities=22 - 27% over two regions of at least 35 amino acid residues) using the BLASTP paradigm of Atschul et al. 1990. Other diseases (such as malignant hyperthermia and hyperkalemic periodic paralysis in humans and the neurodegenerative of mechanosensory neurons in *C. elegans*) arise through mutations in ion channels or receptor proteins. Mutation of the ARMP gene or the *E5-1* gene could affect similar functions and lead to Alzheimer's Disease and other psychiatric and neurological diseases. Based upon this, a test for Alzheimer's Disease can be produced to detect an abnormal receptor or an abnormal ion channel function related to abnormalities that are acquired or inherited in the ARMP gene and its product or in one of the homologous genes such as *E5-1* and their products. This test can be accomplished either *in vivo* or *in vitro* by measurements of ion channel fluxes and/or transmembrane voltage or current fluxes using patch clamp, voltage clamp and fluorescent dyes sensitive to intracellular calcium or transmembrane voltage. Defective ion channel or receptor function can also be assayed by measurements of activation of second messengers such as cyclic AMP, cGMP tyrosine kinases, phosphates, increases in intracellular Ca^{2+} levels, etc. Recombinantly made proteins may also be reconstrued in artificial membrane systems to study ion channel conductance. Therapies which affect Alzheimer's Disease (due to acquired/inherited defects in the ARMP gene or E5-1 gene; due to defects in other pathways leading to this disease such as mutations in APP; and due to environmental

agents) can be tested by analysis of their ability to modify an abnormal ion channel or receptor function induced by mutation in the ARMP gene or in one of its homologues. Therapies could also be tested by their ability to modify the normal function of an ion channel or receptor capacity of the ARMP gene products and its homologues. Such assays can be performed on cultured cells expressing endogenous normal or mutant ARMP genes/gene products or *E5-1* genes/gene products. Such studies can be performed in addition on cells transfected with vectors capable of expressing ARMP, parts of the ARMP gene and gene product, mutant ARMP, *E5-1* gene, parts of the *E5-1* gene and gene product, mutant *E5-1* gene or another homologue in normal or mutant form. Therapies for Alzheimer's Disease can be devised to modify an abnormal ion channel or receptor function of the ARMP gene or *E5-1* gene. Such therapies can be conventional drugs, peptides, sugars, or lipids, as well as antibodies or other ligands which affect the properties of the ARMP or *E5-1* gene product. Such therapies can also be performed by direct replacement of the ARMP gene and/or *E5-1* gene by gene therapy. In the case of an ion channel, the gene therapy could be performed using either mini-genes (cDNA plus a promoter) or genomic constructs bearing genomic DNA sequences for parts or all of the ARMP gene. Mutant ARMP or homologous gene sequence might also be used to counter the effect of the inherited or acquired abnormalities of the ARMP gene as has recently been done for replacement of the mec 4 and deg 1 in *C. elegans* (Huang and Chalfie, 1994). The therapy might also be

directed at augmenting the receptor or ion channel function of the homologous genes such as the E5-1 gene, in order that it may potentially take over the functions of the ARMP gene rendered defective by acquired or inherited defects. Therapy using antisense oligonucleotides to block the expression of the mutant ARMP gene or the mutant *E5-1* gene, coordinated with gene replacement with normal ARMP or *E5-1* gene can also be applied using standard techniques of either gene therapy or protein replacement therapy.

Protein Therapy

Treatment of Alzheimer's Disease can be performed by replacing the mutant protein with normal protein, or by modulating the function of the mutant protein. Once the biological pathway of the ARMP protein has been completely understood, it may also be possible to modify the pathophysiologic pathway (e.g., a signal transduction pathway) in which the protein participates in order to correct the physiological defect.

To replace the mutant protein with normal protein, or with a protein bearing a deliberate counterbalancing mutation it is necessary to obtain large amounts of pure ARMP protein or E5-1 protein from cultured cell systems which can express the protein. Delivery of the protein to the affected brain areas or other tissues can then be accomplished using appropriate packaging or administrating systems.

Gene Therapy

Gene therapy is another potential therapeutic approach in which normal copies of the ARMP gene are introduced into patients to successfully code for normal

protein in several different affected cell types. The gene must be delivered to those cells in a form in which it can be taken up and code for sufficient protein to provide effective function. Alternatively, in some neurologic mutants it has been possible to prevent disease by introducing another copy of the homologous gene bearing a second mutation in that gene or to alter mutation, or use another gene to block its effect.

Retroviral vectors can be used for somatic cell gene therapy especially because of their high efficiency of infection and stable integration and expression. The targeted cells however must be able to divide and the expression of the levels of normal protein should be high because the disease is a dominant one. The full length ARMP gene can be cloned into a retroviral vector and driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest (such as neurons).

Other viral vectors which can be used include adeno-associated virus, vaccinia virus, bovine papilloma virus, or a herpesvirus such as Epstein-Barr virus.

Gene transfer could also be achieved using non-viral means requiring infection *in vitro*. This would include calcium phosphate, DEAE dextran, electroporation, and protoplast fusion. Liposomes may also be potentially beneficial for delivery of DNA into a cell. Although these methods are available, many of these are lower efficiency.

Antisense based strategies can be employed to explore ARMP gene function and as a basis for

therapeutic drug design. The principle is based on the hypothesis that sequence-specific suppression of gene expression can be achieved by intracellular hybridization between mRNA and a complementary antisense species. The formation of a hybrid RNA duplex may then interfere with the processing/transport/translation and/or stability of the target ARMP mRNA. Hybridization is required for the antisense effect to occur, however the efficiency of intracellular hybridization is low and therefore the consequences of such an event may not be very successful. Antisense strategies may use a variety of approaches including the use of antisense oligonucleotides, injection of antisense RNA and transfection of antisense RNA expression vectors. Antisense effects can be induced by control (sense) sequences, however, the extent of phenotypic changes are highly variable. Phenotypic effects induced by antisense effects are based on changes in criteria such as protein levels, protein activity measurement, and target mRNA levels. Multidrug resistance is a useful model to study molecular events associated with phenotypic changes due to antisense effects, since the multidrug resistance phenotype can be established by expression of a single gene mdrl (MDR gene) encoding for P-glycoprotein.

Transplantation of normal genes into the affected area of the patient can also be useful therapy for Alzheimer's Disease. In this procedure, a normal hARMP protein is transferred into a cultivable cell type such as glial cells, either exogenously or endogenously to the patient. These cells are then injected

serotologicially into the disease affected tissue(s). This is a known treatment for Parkinson's disease.

Immunotherapy is also possible for Alzheimer's Disease. Antibodies can be raised to a mutant ARMP protein (or portion thereof) and then administered to bind or block the mutant protein and its deliterious effects. Simultaneously, expression of the normal protein product could be encouraged. Administration could be in the form of a one time immunogenic preparation or vaccine immunization. An immunogenic composition may be prepared as injectables, as liquid solutions or emulsions. The ARMP protein may be mixed with pharmaceutically acceptable excipients compatible with the protein. Such excipients may include water, saline, dextrose, glycerol, ethanol and combinations thereof. The immunogenic composition and vaccine may further contain auxiliary substances such as emulsifying agents or adjuvants to enhance effectiveness. Immunogenic compositions and vaccines may be administered parenterally by injection subcutaneously or intramuscularly.

The immunogenic preparations and vaccines are administered in such amount as will be therapeutically effective, protective and immunogenic. Dosage depends on the route of administration and will vary according to the size of the host.

Similar gene therapy techniques may be employed with respect to the *E5-1* gene.

The above disclosure generally describes the present invention. A more complete understanding can be

obtained by reference to the following specific examples. These examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in the form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Example 1. Development of the
Genetic, Physical "contig"
and Transcriptional Map of the
Minimal Co-Segregating Region

The CEPH Mega YAC and the RPCI PAC human total genomic DNA libraries were searched for clones containing genomic DNA fragments from the AD3 region of chromosome 14q24.3 using oliginucleotide probes for each of the ## SSR marker loci used in the genetic linkage studies as well as ## additional markers depicted in Figure 1a (Albertsen et al., 1990; Chumakov et al., 1992; Ioannu et al., 1994). The genetic map distances between each marker are depicted above the contig, and are derived from published data (NIH/CEPH Collaborative Mapping Group, 1992; Wang, 1992; Weissenbach, J. et al., 1992 Gyapay, G et al., 1994). Clones recovered for each of the initial marker loci were arranged into an ordered series of partially overlapping clones ("contig") using four independent methods. First, sequences representing the ends of the YAC insert were isolated by inverse PCR (Riley et al., 1990), and hybridized to Southern blot panels containing restriction digests of DNA from all of the YAC clones bearing overlapping sequences. Second,

inter-Alu PCR was performed on each YAC, and the resultant band patterns were compared across the pool of recovered YAC clones in order to identify other clones bearing overlapping sequences (Bellamne-Chartelot et al., 1992; Chumakov et al; 1992). Third, to improve the specificity of the Alu-PCR fingerprinting, we restricted the YAC DNA with HaeIII or RsaI, amplified the restriction products with both Alu and L1H consensus primers, and resolved the products by polyacrylamide gel electrophoresis. Finally, as additional STSs were generated during the search for transcribed sequences, these STSs were also used to identify overlaps. The resultant contig was complete except for a single discontinuity between YAC932C7 bearing *D14S53* and YAC746B4 containing *D14S61*. The physical map order of the STSs within the contig was largely in accordance with the genetic linkage map for this region (NIH/CEPH Collaborative Mapping Group, 1992; Wang, Z., Webber, J.L., 1992; Weissenbach, J. et al., 1992; Gyapay, G. et al., 1994). However, as with the genetic maps, we were unable to unambiguously resolve the relative order of the loci within the *D14S43/D14S71* cluster and the *D14S76/D14S273* cluster. PAC1 clones suggest that D14S277 is telomeric to D14S268, whereas genetic maps have suggested the reverse order. Furthermore, a few STS probes failed to detect hybridization patterns in at least one YAC clone which, on the basis of the most parsimonious consensus physical map and from the genetic map, would have been predicted to contain that STS. For instance, the D14S268 (AFM265) and RSCAT7 STSs are absent from YAC788H12 (Figure 3).

Because these results are reproducible, and occurred with several different STS markers, these results most likely reflect the presence of small interstitial deletions with one of the YAC clones.

Example 2. Cumulative two-point lod scores for chromosome 14q24.3 markers.

Genotypes of each polymorphic microsatellite marker locus were determined by PCR from 1000ng of genomic DNA of all available affected and unaffected pedigree members as previously described (St. George-Hyslop, P et al, 1992) using primer sequences specific for each microsatellite locus (Weissenbach, J et al., 1992; Gyapay, G et al., 1994). The normal population frequency of each allele was determined using spouses and other neurologically normal subjects from the same ethnic groups, but did not differ significantly from those established for mixed Caucasian populations (Weissenbach, J. et al., 1992; Gyapay, G. et al., 1994). The maximum likelihood calculations assumed an age of onset correction, marker allele frequencies derived from published series of mixed Caucasian subjects, an estimated allele frequence for the AD3 mutation of 1:1000 as previously described (St. George-Hyslop, P. et al., 1992). The analyses were repeated using equal marker allele frequencies, and using phenotype information only from affected pedigree members as previously described to ensure that inaccuracies in the estimated parameters used in the maximum likelihood calculations did not misdirect the analyses (St. George-Hyslop, P. et al., 1992). These supplemental analyses did not significantly alter either the evidence

supporting linkage, or the discovery of recombination events.

Example 3. Haplotypes between flanking markers segregated with AD3 in FAD pedigrees

Extended haplotypes between the centromeric and telomeric flanking markers on the parental copy of chromosome 14 segregating with AD3 in fourteen early onset FAD pedigrees (pedigrees NIH2, MGH1, Tor1.1, FAD4, FAD1, MEX1, and FAD2 show pedigree specific lod scores ≥ +3.00 with at least one marker between D14S258 and D14S53). Identical partial haplotypes (boxed) are observed in two regions of the disease bearing chromosome segregating in several pedigrees of similar ethnic origin. In region A, shared alleles are seen at D14S268 ("B": allele size = 126 bp, allele frequence in normal Caucasians = 0.04; "C": size = 124 bp, frequency = 0.38); D14S277 ("B": size = 156 bp, frequency = 0.19; "C": size - 154 bp, frequency = 0.33); and RSCAT6 ("D": size = 111bp, frequency 0.25; "E" size = 109 bp, frequency = 0.20; "F" size = 107 bp, frequency = 0.47). In region B, alleles of identical size are observed at D14S43 ("A": size = 193bp, frequency = 0.01; "D": size 187 bp, frequency = 0.12; "E" size = 185bp, frequency = 0.26; "I" size = 160 bp, frequency = 0.38); D14S273 ("3": size = 193 bp, frequency = 0.38; "4" size = 191 bp, frequency = 0.16; "5": size = 189 bp, frequency = 0.34; "6": size = 187 bp, frequency = 0.02) and D14S76 ("1": size = bp, frequency = 0.01; "5": size = bp, frequency = 0.38; "6": size = bp, frequency = 0.07, "9": size = bp, frequency = 0.38). The ethnic origins of each pedigree are abbreviated as: Ashk = Askenazi

Jewish; Ital = Southern Italian; Angl = Anglo-Saxon-Celt; FrCan = French Canadian; Jpn = Japanese; Mex = Mexican Caucasian; Ger = German; Am = American Caucasian. The type of mutation detected is depicted by the amino acid substitution and putative condon number or by ND where no mutation has been detected because a comprehensive survey has not been undertaken due to the absence of a source of mRNA for RT-PCR studies.

Example 4. Recovery of transcribed sequences from the AD3 interval.

Putative transcribed sequences encoded in the AD3 interval were recovered using either a direct hybridization method in which short cDNA fragments generated from human brain mRNA were hypridized to immobilized cloned genomic DNA fragments (Rommens, JM et al., 1993). The resultant short putatively transcribed sequences were used as probes to recover longer transcripts from human brain cDNA libraries (Stratagene, La Jolla). The physical locations of the original short clone and of the subsequently acquired longer cDNA clones were established by analysis of the hybridization pattern generated by hybridizing the probe to Southern blots containing a panel of EcoRI digested total DNA samples isolated from individual YAC clones within the contig. The nucleotide sequence of each of the longer cDNA clones was determined by automated cycle sequencing (Applied Biosystems Inc., CA), and compared to other sequences in nucleotide and protein databases using the blast algorithm (Atschul, SF et al., 1990). Accession numbers for the transcribed sequences in this report are

L40391, L40392, L40393, L40394, L40395, L40396, L40397, L40398, L40399, L40400, L40401, L40402, and L40403.

Example 5. Locating mutations in the ARMP gene using restriction enzymes.

The presence of Ala 246 Glu mutation which creates a DdeI restriction site was assayed in genomic DNA by PCR using the end labelled primer 849 (5'-atctccggcaggcatatct-3') SEQ ID NO:129 and the unlabelled primer 892 (5'-tgaaatcacagccaagatgag-3') SEQ ID NO:130 to amplify an 84bp genomic exon fragment using 100ng of genomic DNA template, 2mM $MgCl_2$, 10 pMoles of each primer, 0.5U Taq polymerase, 250 uM dNTPs for 30 cycles of 95°C x 20 seconds, 60°C X 20 seconds, 72°C X 5 seconds. The products were incubated with an excess of DdeI for 2 hours according to the manufacturers protocol, and the resulting restriction fragments were resolved on a 6% nondenaturing polyacrylamide gel and visualized by autoradiography. The presence of the mutation was inferred from the clevage of the 84bp fragment due to the presence of a DdeI restriction site. All affected members of the FAD1 pedigree (filled symbols) and several at-risk members ("R") carried the DdeI site. None of the obligate escapees (those individuals who do not get the disease, age > 70 years), and none of the normal controls carried the DdeI mutation.

Example 6. ~~Location~~ Locating mutations in the ARMP gene using allele specific oligonucleotides.

The presence of the Cys 410 Tyr mutation was assayed using allele specific oligonucleotides. 100ng of genomic DNA was amplified with the exonic sequence

primer 885 (5'-tggagactggaacacaac-3') SEQ ID NO:127 and the opposing intronic sequence primer 893 (5'-gtgtggccagggtagagaact-3') SEQ ID.NO:128 using the above reaction conditions except 2.5 mM $MgCl_2$, and cycle conditions of 94°C X 20 seconds, 58°C X 20 seconds, and 72°C for 10 seconds). The resultant 216bp genomic fragment was denatured by 10-fold dilution in 0.4M NaOH, 25mM EDTA, and was vacuum slot-blotted to duplicate nylon membranes. The end-labelled "wild-type" primer 890 (5'-ccatagcctgtttcgtagc-3') SEQ ID NO:131 and the end-labelled "mutant" primer 891 (5'-ccatagcctAtttcgtagc-3') SEQ ID NO:132 were hybridized to separate copies of the slot-blot filters in 5 X SSC, 5X Denhardt's, 0.5% SDS for 1 hour at 48°C, and then washed successively in 2 X SSC at 23°C and 2 X SSC, 0.1% SDS at 50°C and then exposed to X-ray film. All testable affected members as well as some at-risk members of the AD3 (shown) and NIH2 pedigrees (not shown) possessed the Cys 410 Tyr mutation. Attempts to detect the Cys 410 Try mutation by SSCP revealed that a common intronic sequence polymorphism migrated with the same SSCP pattern.

Example 7. Northern hybridization demonstrating the expression of ARMP protein mRNA in a variety of tissues.

Total cytoplasmic RNA was isolated from various tissue samples (including heart, brain, and different regions of placenta, lung, liver, skeletal muscle, kidney and pancreas) obtained from surgical pathology using standard procedures such as CsCl purification. The RNA was then electrophoresed on a formaldehyde gel to permit size fractionation.

The nitrocellulose membrane was prepared and the RNA was then transferred onto the membrane. ^{32}P-labelled cDNA probes were prepared and added to the membrane in order for hybridization between the probe the RNA to occur. After washing, the membrane was wrapped in plastic film and placed into imaging cassettes containing X-ray film. The autoradiographs were then allowed to develop for one to several days. The positions of the 28S and 18S rRNA bands are indicated. Sizing was established by comparison to standard RNA markers. Analysis of the autoradiographs revealed a prominent band at 3.0kb in size. These northern blots demonstrated the ARMP gene is expressed in all of the tissues examined.

Example 8: Eukaryotic and Prokaryotic Expression Vector Systems

Eukaryotic and prokaryotic expression systems have been generated using two different classes of ARMP nucleotide cDNA sequence inserts. In the first class, termed full-length constructs, the entire ARMP cDNA sequence was inserted into the expression plasmid in the correct orientation, and included both the natural 5' UTR and 3' UTR sequences as well as the entire open reading frame. The open reading frames bear a nucleotide sequence cassette which allows either the wild type open reading frame to be included in the expression system or alternatively, single or a combination of double mutations can be inserted into the open reading frame. This was accomplished by removing a restriction fragment from the wild type open reading frame using the enzymes NarI and PflmI and replacing it

with a similar fragment generated by reverse transcriptase PCR and which bears the nucleotide sequence encoding either the Met146Leu mutation or the Hys163Arg mutation. A second restriction fragment was removed from the wild type normal nucleotide sequence for the open reading frame by cleavage with the enzymes PflmI and NcoI and replaced with restriction fragments bearing either the nucleotide sequence encoding the Ala246Glu mutation, or the Ala260Val mutation or the Ala285Val mutation or the Leu286Val mutation, or the Leu392Val mutation, or the Cys410 Tyr mutation. Finally, a third variant bearing combinations of either the Met146Leu or His163Arg mutations in tandem with the remaining mutations by linking the NarI-PflmI fragment bearing these mutations and the PflmI-NcoI fragment bearing the remaining mutations.

A second variant of cDNA inserts bearing wild type or mutant cDNA sequences was constructed by removing from the full-length cDNA the 5' UTR and part of the 3' UTR sequences. The 5' UTR sequence was replaced with a synthetic oligonucleotide containing a KpnI restriction site and a Kozak initiation site (oligonucleotide 969: ggtaccgccaccatgacagaggtacctgcac) SEQ ID NO:139. The 3' UTR was replaced with an oligonucleotide corresponding to position 2566 of the cDNA and bears an artificial EcoRI site (oligonucleotide 970:gaattcactggctgtagaaaaagac) SEQ ID NO:140. Mutant variants of this construct were then made by inserting the same mutant sequences described above at the NarI-PflmI fragment, and at the PsImI-NcoI sites described above.

For eukaryotic expressions, these various cDNA constructs bearing wild type and mutant sequences were cloned into the expression vector pZeoSV (invitrogen). For prokaryotic expression, two constructs were made using the gluthathione S-transferase fusion vector pGEX-kg. The inserts which have been attached to the GST fusion nucleotide sequence are the same nucleotide sequence described above generated with the oligonucleotide primers 969, SEQ ID NO:139 and 970, SEQ ID NO:140, bearing either the normal open reading frame nucleotide sequence or bearing a combination of single and double mutations as described above. This construct allows expression of the full-length protein in mutant and wild type variants in prokaryotic cell systems as a GST fusion protein which will allow purification of the full-length protein followed by removal of the GST fusion product by thrombin digestion. The second prokaryotic cDNA construct was generated to create a fusion protein with the same vector, and allows the production of the amino acid sequence corresponding to the hydrophillic acid loop domains between TM6 and TM7 of the full-length protein, as either a wild type nucleotide sequence (thus a wild type amino acid sequence for fusion proteins) or as a mutant sequence bearing either the Ala285Val mutation, or the Leu286Val mutation, or the Leu392Val mutation. This was accomplished by recovering wild type or mutant sequence from appropriate sources of RNA using the oligonucleotide primers 989:ggatccggtccacttcgtatgctg SEQ ID NO:141, and 990:tttttgaattcttaggctatggttgtgttcca SEQ ID NO:142. This allows cloning of the appropriate

mutant or wild type nucleotide sequence corresponding to the hydrophillic acid loop domain at the BamHI and the EcoRI sites within the pGEX-KG vector.

These prokaryotic expression systems allow the holo-protein or various important functional domains of the protein to be recovered as fusion proteins and then used for binding studies, structural studies, functional studies, and for the generation of appropriate antibodies.

Example 9: Identification of Three New Mutations in the ARMP Gene.

Three novel mutations have been identified in subjects affected with early onset Alzheimer's Disease. All of these mutations co-segregate with the disease, and are absent from at least 200 normal chromosomes. The three mutations are as follows: a substitution of C by T at position 1027 which results in the substitution of alanine 260 for valine; substitution of C by T at position 1102, which results in the substitution of alanine at 285 by valine; and substitution of C by G at position 1422 which results in the substitution of leucine 392 by valine. Significantly, all of these mutations occur within the acidic hydrophillic loop between putative TM6 and TM7. Two of the mutations (A260V; A285V) and the L286V mutation are also located in the alternative spliced domain.

The three new mutations, like the other mutations, can be assayed by a variety of strategies (direct nucleotide sequencing, Allele specific oligos, ligation polymerase chain reaction, SSCP, RFLPs) using RT-PCR products representing the mature mRNA/cDNA sequence or

genomic DNA. We have chosen allele specific oligos. For the A260V and the A285V mutations, genomic DNA carrying the exon can be amplified using the same PCR primers and methods for the L286V mutation. PCR products were then denatured and slot blotted to duplicate nylon membranes using the slot blot protocol described for the C410T mutation.

The Ala260Val mutation was scored by these blots by using hybridization with end-labeled allele-specific oligonucleotides corresponding to the wild type sequence (994:gattagtggttgttttgtg) SEQ ID NO:143 or the mutant sequence (995:gattagtggctgttttgtg) SEQ ID NO:144 by hybridization at 48°C followed by a wash at 52° in 3X SSC buffer containing 0.1% SDS. The Ala285Val mutation was scored on these slot blots as described above but using instead the allele-specific oligonucleotides for the wild type sequence (1003:tttttccagctctcattta) SEQ ID NO:145 or the mutant primer (1004:tttttccagttctcattta) SEQ ID NO:146 at 48°C followed by washing at 52°C as above except that the wash solution was 2X SSC.

The Leu392Val mutation was scored by amplification of the exon from genomic DNA using primers 996 (aaacttggattgggagat) SEQ ID NO:167 and 893 (gtgtggccagggtagagaact) SEQ ID NO:128 using standard PCR buffer conditions excepting that the magnesium concentration was 2mM and cycle conditions were 94°C time 10 seconds, 56°C times 20 seconds, and 72°C for 10 seconds. The result 200 based pair genomic fragment was denatured as described for the Cys410Tyr mutation and slot-blotted in duplicate to nylon membranes. The

presence or absence of the mutation was then scored by differential hybridization to either a wild type end-labelled oligonucleotide (999:tacagtgttctggttggta) SEQ ID NO:148 or with an end-labeled mutant primer (100:tacagtgttgtggttggta) SEQ ID NO:149 by hybridization at 45°C and then successive washing in 2X SSC at 23° and then at 68°C.

Example 10: Polyclonal Antibody Production

Peptide antigens were synthesized by solid-phase techniques and purified by reverse phase high pressure liquid chromatography. Peptides were covalently linked to keyhole limpet hematoxylin (KLH) via disulfide linkages that were made possible by the addition of a cystein residue at the peptide C-terminus. This additional residue does not appear normally in the protein sequence and was included only to facilitate linkage to the KLH molecule. A total of three rabbits were immunized with peptide-KLH complexes for each peptide antigen and were then subsequently give booster injections at seven day intervals. Antisera were collected for each peptide and pooled and IgG precipitated with ammonium sulfate. Antibodies were then affinity purified with Sulfo-link agarose (Pierce) coupled with the appropriate peptide. This final purification is required to remove non-specific interactions of other antibodies present in either the pre- or post-immune serum.

The specific sequences to which we have raised antibodies are:

Polyclonal antibody 1: NDNRERQEHNDRRSL (C)-residues 30-45
SEQ ID NO:168

Polyclonal antibody 2: KDGQLIYTPFTEDTE (C)-residues 109-120
SEQ ID NO:169

Polyclonal antibody 3: EAQRRVSKNSKYNAE (C)-residues 304-319
SEQ ID NO:170

Polyclonal antibody 4: SHLGPHRSTPESRAA (C)-residues 346-360
SEQ ID NO:171

The non-native cysteine residue is indicated at the C-terminal by (C). These sequences are contained within various predicted domains of the protein. For example, antibodies 1,3, and 4 are located in potentially functional domains that are exposed to the aqueous media and may be involved in binding to other proteins critical for the development of the disease phenotype. Antibody 2 corresponds to a short linking region situated between the predicted first and second transmembrane helices.

Example 11: Identification of two mutations in E5-1 gene.

RT-PCR products corresponding to the *E5-1* ORF were generated from RNA of lymphoblasts or frozen post-mortem brain tissue using oligonucleotide primer pairs 1021:5'-cagaggatggagagaatac SEQ ID NO:172 and 1018:5'-ggctccccaaaactgtcat SEQ ID NO:173 (product = 888 bp); and 1071:5'-gccctagtgttcatcaagta SEQ ID NO:174 and 1022:5'-aaagcgggagccaaagtc SEQ ID NO:175 (product = 826 bp) by PCR using 250 μMol dNTPs, 2.5 mM MgC12, 10pMol oligunucleotides in 10 μl cycled for 40 cycles of 94°C X 20 seconds, 58°C X 20 seconds, 72°C X 45 seconds. The PCR products were sequenced by automated cycle sequencing (ABI, Foster City, A) and the fluorescent chromatograms were scanned for heterozygous nucleotide substitutions by direct inspection and by the Factura

(ver 1.2.0) and Sequence Navigator (ver 1.0.1b15) software packages (data not shown).

Asn141Ile: the A→T substitution at nucleotide 787 creates a BclI restriction site. The exon bearing this mutation was amplified from 100 ng of genomic DNA using 10pMol of oligonucleotides 1041: 5'-cattcactaggacacacc SEQ ID NO:163 (end-labelled) and 1042: 5'-tgtagagcaccaccaaga SEQ ID NO:164 (unlabelled), and PCR reaction conditions similar to those described below for the Met239Val. 2μl of the PCR product was restricted to BclI (NEBL, Beverly, MA) in 10 μl reaction volume according to the manufacturers' protocol, and the products were resolved by non - denaturing polyacrylamide gel electrophoresis. In subjects with wild type sequences, the 114 bp PCR product is cleaved into 68 bp and 46 bp fragments. Mutant sequences cause the product to be cleaved into 53 bp, 46 bp and 15 bp.

Met239Val: The A→G substitution at nucleotide 1080 deletes a NlaIII restriction site, allowing the presence of the Met239Val mutation to be detected by amplification from 100 ng of genomic DNA using PCR (10 pMol oligonucleotides 1034:5'-gcatggtgtgcatccact SEQ ID NO:165, 1035:5'-ggaccactctgggaggta SEQ ID NO:166; 0.5 U Taq polymerase, 250μM dNTPS, 1μCi alpha ^{32}P-dCTP, 1.5 mM $MgCI_2$, 10μl volume; 30 cycles of 94°C X30 seconds, 58°C X 20 seconds, 72°C X 20 seconds) to generate a 110 bp product. 2μl of the PCR reaction were diluted to 10μl and restricted with 3 U of NlaIII (NEBL, Beverly MA) for 3 hours. The restriction products were resolved by non-denaturing polyacrylamide gel electrophoresis and

visualized by autoradiography. Normal subjects show cleavage products of 55, 35, 15 and 6bp, whereas the mutant sequence gives fragments of 55, 50 and 6 bp.

Although preferred embodiments of the invention have been described herein in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the appended claims.

F:\DOCS1\TMP\9.0\153197.DOC

RECOMBINATION FRACTION (θ)							
LOCUS	0.00	0.05	0.10	0.15	0.20	0.30	0.40
D14S63	$-\infty$	1.54	3.90	4.38	4.13	2.71	1.08
D14S258	$-\infty$	21.60	19.64	17.19	14.50	8.97	3.81
D14S77	$-\infty$	15.18	15.53	14.35	12.50	7.82	2.92
D14S71	$-\infty$	15.63	14.14	12.19	10.10	5.98	2.39
D14S43	$-\infty$	19.36	17.51	15.27	12.84	7.80	3.11
D14S273	$-\infty$	12.30	11.52	10.12	8.48	5.04	1.91
D14S61	$-\infty$	26.90	24.92	22.14	18.98	12.05	5.07
D14S53	$-\infty$	11.52	11.41	10.39	8.99	5.73	2.51
D14S48	$-\infty$	0.50	1.05	1.14	1.04	0.60	0.18

TABLE 1

	PEDIGREE ID													
LOCUS	N1B2	FaD3	TUR1.1	FaD4	RB	FaD1	BIG12	BOW	COOK	603	Tor42	QUE	MEX1	FAD2
D14S83	1	4	7	4		5						[illegible]	9	2
D14S258	6	6	8	7	4	5	5	6	6		7	6	7	6
D14S268	C	C	B	B	C	C	C	C	C	C	C	B	C	C
D14S277	C	C	C	C	C	C	C	C	C	A	A	C	B	B
D14S786	D	D	E	E	F	E	E	D/F	E	E	E	E	F	D
D14S77	Y	Y	K	S		P	P	X	H		C	U	F	A
D14S78	7	7	1	5	7	7		6	7		3	7	2	6
D14S43	A	A	I	I	I	E	D	I	I		C	I	D	C
D14S273	6	6	3	5	5	4	4	4	6		6	6	5	3
D14S76	5	5	5	5	5	6	9	9			9	1	5	5
D14S61	E	E	G	F		I					D		L	F
D14S53	F	F	C	F	F	J	C	F	E		J	D	F	F
ETHNIC ORIGIN	Ashk	Ashk	Ital	Ital	Ital	Angl	Angl	Angl	Angl	Amer	FrCan	FrCan	Mex	Ger
MUTATION	C410Y	C410Y	M146L	M146L	ND	A246E	ND	ND	ND	H163R	H163R	ND	ND	L286V

TABLE 2

```
No.  Target File                        Key   Target   Overlap    Match   Similarities
  1  marmp.con/long(Frame 1)              1        1       467      465     Percentage
                                                                                99.57%

         1        10        20        30        40        50        60        70
Human N- MTELPAPLSYFQNAQMSEDNHLSNTVRSQNDNRERQEHNDRRSLGHPEPLSNGRPQGNSRQVVEQDEEED
         **********************************************************************
Mouse N- MTEIPAPLSYFQNAQMSEDSHSSSAIRSQNDSQERQQHDRQRLDNPEPISNGRPQSNSRQVVEQDKEED
         1        10        20        30        40        50        60        70

        71        80        90       100       110       120       130       140
         EELTLKYGAKHVIMLFVPVTLCMVVVVATIKSVSFYTRKDGQLIYTPFTEDTETVGQRALHSILNAAIMI
         **********************************************************************
         EELTLKYGAKHVIMLFVPVTLCMVVVVATIKSVSFYTRKDGQLIYTPFTEDTETVGQRALHSILNAAIMI
        71        80        90       100       110       120       130       140

       141       150       160       170       180       190       200       210
         SVIVVMTILLVVLYKYRCYKVIHAWLIISSLLLLFFFSFIYLGEVFKTYNVAVDYITVALLINNLGVVGM
         **********************************************************************
         SVIVIMTILLVVLYKYRCYKVIHAWLIISSLLLLFFFSFIYLGEVFKTYNVXVDYVTVALLIWNNGVVGM
       141       150       160       170       180       190       200       210

       211       220       230       240       250       260       270       280
         ISIHWKGPLRLQQAYLIMISALMALVFIKYLPEWTANLILAVISVYDLVAVLCPKGPLRMLVETAQERNE
         **********************************************************************
         IAIHWKGPLRLQQAYLIMISALNALVFIKYLPEWTAWLILAVISVYDLVAVLCPKGPLRMLVETAQERNE
       211       220       230       240       250       260       270       280

       281       290       300       310       320       330       340       350
         TLFPALIYSSTMVWLVNMAEGDPEAQRRVSKNSKYNAESTERESQDTVAENDDGGFSEEWEAQRDSHLGP
         ***************************** ** *************************************
         TLFPALIYSSTMVWLVNMAEGDPEAQRRVPKNPKYNTQRAERETQDSGSGNDDGGFSKEWEAQRDSHLGP
       281       290       300       310       320       330       340       350

       351       360       370       380       390       400       410       420
         HRSTPESRAAVQELSSSILAGEDPEERGVKLGLGDPIFYSVLVGKASATASGDWNTTIACFVAILIGLCL
         **********************************************************************
         HRSTPESRAAVQELSGSILTSEDPEERGVKLGLGDFIFYSVLVGKASATASGDWNTTIACXVAILIGLCL
       351       360       370       380       390       400       410       420

       421       430       440       450       460
         TLLLLAIFKKALPALPISITFGLVFYFATDYLVQPFMDQLAFHQFYI  -C    SEQ ID NO: 2
         ***********************************************
         XLLLLAIYKKGXPAXPISITFGFVFXFATDYLVQPFMDQLAFHQFYI  -C    SEQ ID NO: 4
       421       430       440       450       460
```

TABLE 3

HUMAN ARMP FUNCTION DOMAINS

Domains (Amino Acid Residue)						Functional Characteristic
82	-	100	AA			Hydrophobic
132	-	154	AA			Hydrophobic
164	-	183	AA			Hydrophobic
195	-	213	AA			Hydrophobic
221	-	238	AA			Hydrophobic
244	-	256	AA			Hydrophobic
281	-	299	AA			Hydrophobic
404	-	428	AA			Hydrophobic
431	-	449	AA			Hydrophobic
115	-	119	AA	(YTPF)	SEQ ID NO:161	Phosphorylation Site
353	-	356	AA	(STPC)	SEQ ID NO:162	Phosphorylation Site
300	-	385	AA			Acid Rich Domain Possible Metal Binding Domain

ANTIGENIC SITES INCLUDING AMINO ACID RESIDUES

27	-	44
46	-	48
50	-	60
66	-	67
107	-	111
120	-	121
125	-	126
155	-	160
185	-	189
214	-	223
220		230
240	-	245
267	-	269
273	-	282
300	-	370
400	-	420

TABLE 4

MUTATION	ENZYME (effect of mutation)	AMPLIFICATION 0440 #1	AMPLIFICATION 0440 #2	ALLELE-SPECIFIC 0440
M146LEU	Bsphl (destroy)	910 (170-S182F) TCACAGAAGATACCG AGACT (SEQ ID NO:176)	911 (170-S182) R CCCAACCATAAGAAG AACAG (SEQ ID NO:177)	
MIS 164 Ary	Nla III (destroy)	927 (intronic) TCTGTACTTTTTAAG GGTTGTG (SEQ ID NO:178)	928 ACTTCAGAGTAATTC ATCANCA (SEQ ID NO:179)	
Ala 246	Dlc I (create)	849 * GACTCCAGCAGGCAT ATCT (SEQ ID NO:80)	892 TGAAATCACAGCCAA GATGAG (SEQ ID NO:130)	
Leu 286 Val.	Pvu II (create)	952 GATGAGACAAGTNCC NTGAA (SEQ ID NO:181) 945 TTAGTGGCTGTTTNG TGTCC (SEQ ID NO:182)	951 CACCCATTTACAAGT TTAGC (SEQ ID NO:183)	
Cys 410 Tys	Allele specific ligo	893 GTGTGGCCAGGGTAG AGAACT (SEQ ID NO:128)	885 TGGAGACTGGAACAC AAC (SEQ ID NO:127)	CCATAGCCTGTTTCGTAGC (SEQ ID NO:131) 890 = WT CCATAGCCTATTTCGTAGC (SEQ ID NO:132) 891 = MUT

TABLE 5

POSITION OF EXONS AND INTRON-EXON BOUNDARIES OF THE ARMP GENE

cDNA/mRNA SEQUENCE		CORRESPONDING GENOMIC SEQUENCE	
ARMP (917 ver)	Transcipt ID CC44 ver	Genomic sequence file ID & position of exon	Comments
1-113bp	N/A	917-936.gen @ 731-834bp	Alternate 5'UTR
N/A	1-422bp	917-936.gen @ 1067-1475bp	Alternate 5'UTR
114-195bp	423-500bp	932-943.gen @ 589-671bp	
196-335bp	501-632bp	932-943.gen @ 759-899bp	12bp Variably spliced
337-586bp	633-883bp	901-912.gen @ 787-1037bp	
587-730bp	884-1026bp	910-915.gen @ 1134-1278bp	M146L mutation
731-795bp	1027-1092bp	925-913.gen @ 413-478bp	H163R mutation
796-1017bp	1093-1314bp	849-892.gen @ 336-558bp	A246E mutation
1018-1116bp	1315-1413bp	951-952.gen @ 312-412bp	L286V mutation, variable spl
1117-1204bp	1414-1501bp	983-1011.gen @ 61-149bp	
1205-1377bp	1502-1674bp	874-984.gen @ 452-625bp	
1378-1497bp	1674-1794bp	885-1012.gen @ 431-550bp	C410Y mutation
1498-2760bp	1795-3060bp	930-919.gen @ -10bp-end	last AA, STOP, 3'UTR

TABLE 6

MUTATIONS AND POLYMORPHISMS IN THE ARMP GENE

Nucleotide # in ARMP.UPD	Amino acid # in ARMP.PRO	Comment
A->C_{684}	Met146Leu	Pathogenic. Unique to AD affected.
A->G_{736}	His163Arg	"
C->A_{985}	Ala246Glu	"
C->T_{1027}	Ala260Val	"
C->T_{1102}	Ala285Val	"
C->G_{1104}	Leu286Val	"
C->G_{1422}	Leu392Val	"
G->A_{1477}	Cys410Tyr	"
G->T_{863}	Phe205Leu	Polymorphism in normals
C->A_{1700}	non-coding	3'UTR polymorphism
G->A_{2601}	non-coding	"
delC_{2620}	non-coding	"

TABLE 7

```
E5-1   1   MLTFMASDSEEEVCDERTSLMSAESPTPRSC·QEGRQGPEDGE··NTAQWRSQENEED   55
S182   1   ··················MT·ELPAPLSYFQNA·QMSEDNHLSNTV··RSQ·N··D      31

E5-1   56  G·E··EDPDRYVCS·GVP·····GRPPGL·····E······EELTLKYGAKHVIMLF    92
S182   51  NRERQEHNDRR··SLGHPEPLSNGRPQGNSRQVVEQDEEEDEELTLKYGAKHVIMLF    86

                                                         I
E5-1   93  VPVTLCMIVVVATIKSVRFYTEKNGQLIYTPFTEDTPSVGQRLLNSVLNTLIMISVI   149
S182   87  VPVTLCMVVVVATIKSVSFYTRKDGQLIYTPFTEDTETVGQRALHSILNAAIMISVI   143

E5-1   150 VVMTIFLVVLYKYRCYKFIHGWLIMSSLMLLFLFTYIYLGEVLKTYNVAMDYPTL·L   205
S182   144 VVMTILLVVLYKYRCYKVIHAWLIISSLLLLFFFSFIYLGEVFKTYNVAVDYITVAL   200
             L              R

                                         V
E5-1   206 LTVWNFGAVGMVCIHWKGPLVLQQAYLIMISALMALVFIKYLPEWSAWVILGA·ISV   261
S182   201 LI·WNFGVVGMISIHWKGPLRLQQAYLIMISALMALVFIKYLPEWTAWLILAVISV    255
                                                        E

E5-1   262 YDLVAVLCPKGPLRMLVETAQERNEPIFPALIYSSAMVWTVGMAKLDP······S··   310
S182   256 YDLVAVLCPKGPLRMLVETAQERNETLFPALIYSSTMVWLVNMAEGDPEAQRRVSKN   312
                                    VV

E5-1   311 SQGAL·······QLPY···DP·····EME·EDSYDSF·GEP··SYPE····VFEPPLT   345
S182   301 SKYNAESTERESQDTVAENDDGGFSE·EWEAQRDSHLG·PHRSTPESRAAVQE··LS   365

E5-1   346 GYP··GEELEEEEERGVKLGLGDFIFYSVLVGKAAATGSGDWNTTLACFVAILIGLC   400
S182   366 SSILAGEDP···EERGVKLGLGDFIFYSVLVGKASATASGDWNTTIACFVAILIGLC   420
                                   V                Y

E5-1   401 LTLLLLAVFKKALPALPISITFGLIFYFSTDNLVRPFMDTLASHQLYI*   448   SEQ ID NO:138
S182   421 LTLLLLAIFKKALPALPISITFGLVFYFATDYLVQPFMDQLAFHQFYI*   467   SEQ ID NO:2
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TABLE 8